Physiological roles of endogenous neurosteroids at α2 subunit-containing $GABA_A$ receptors

Elizabeth Jane Durkin

A thesis submitted to University College London for the degree of Doctor of Philosophy

October 2012

Department of Neuroscience, Physiology and Pharmacology

University College London

Gower Street

London

WC1E 6BT

Declaration

I, Elizabeth Durkin, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Abstract

Neurosteroids are important endogenous modulators of the major inhibitory neurotransmitter receptor in the brain, the γ-amino-butyric acid type A ($GABA_A$) receptor. They are involved in numerous physiological processes, and are linked to several central nervous system disorders, including depression and anxiety. The neurosteroids allopregnanolone and allo-tetrahydro-deoxy-corticosterone (THDOC) have many effects in animal models (anxiolysis, analgesia, sedation, anticonvulsion, antidepressive), suggesting they could be useful therapeutic agents, for example in anxiety, stress and mood disorders.

Neurosteroids potentiate GABA-activated currents by binding to a conserved site within α subunits. Potentiation can be eliminated by hydrophobic substitution of the $α1^{Q241}$ residue (or equivalent in other α isoforms). Previous studies suggest that α2 subunits are key components in neural circuits affecting anxiety and depression, and that neurosteroids are endogenous anxiolytics. It is therefore possible that this anxiolysis occurs via potentiation at α2 subunit-containing receptors. To examine this hypothesis, $α2^{Q241M}$ knock-in mice were generated, and used to define the roles of α2 subunits in mediating effects of endogenous and injected neurosteroids.

Biochemical and imaging analyses indicated that relative expression levels and localization of $GABA_A$ receptor α1-α5 subunits were unaffected, suggesting the knock-in had not caused any compensatory effects. Electrophysiological characterization of cells in hippocampal and nucleus accumbens brain slices revealed faster-decaying inhibitory synaptic transmission in $α2^{Q241M}$ mice. Furthermore, the response to applied THDOC was markedly reduced compared to wild-type cells. α2 subunits therefore formed a major component of synaptic $GABA_A$ receptors in these areas.

The $α2^{Q241M}$ knock-ins showed greater anxiety levels in two classical rodent anxiety paradigms (light-dark box and elevated plus maze), consistent with endogenous neurosteroids mediating anxiolysis via α2-type $GABA_A$ receptors. In addition, the anxiolytic response to injected THDOC is impaired by the $α2^{Q241M}$ mutation, which would identify the α2 isoform as an appropriate target for generating receptor subtype-selective neurosteroid therapeutics for anxiety disorders.

Acknowledgements

I will forever be indebted to the many people who have helped me along my PhD journey, especially to Prof. Trevor Smart, for allowing me to work on such a challenging and exciting project, and for his unending support, guidance and enduring positivity. This project would not have been possible without the financial support of the MRC, and helpful advice from my thesis committee, Stuart Cull-Candy, Stephen Nurrish and Antonella Riccio.

Thank you to Mike Lumb, for generating the transgenic mouse strain, together with Steve Moss and GenOway. For generously providing lab space and loaning me the equipment necessary for the behavioural analyses, I am grateful to Clare Stanford and Stephen Hunt. I would also like to thank Clare, and members of her lab, especially Ruth Weir and Ewelina Grabowska, for teaching me behavioural techniques, and helping me interpret results from these experiments. Thank you also to Martin Stocker for allowing access to his microtome.

Thanks to all members of the Smart lab, past and present, for creating an excellent environment to work in, and being a great source of fun and of encouragement through the inevitable experimental difficulties. I would particularly like to thank Damian and Phil, for their excellent teaching and support, and for being on hand whenever my rig misbehaved! Also, thanks to Saad for sharing his imaging expertise, and PhD pep talks.

I would also like to take this opportunity to thank all of the Durkin ‘clan’ – above all, Mum, Dad, Rob, Billy and Pat – for reminding me that there is life outside of my PhD, and whose love and support has given me the drive to make it through my academic career to date. I am particularly grateful to my PhD buddies who have shared in this rollercoaster ride, especially Sinead and Kasia, who have always been there for a chat over coffee.

Finally, the biggest of thanks is owed to Stuart for his patience, constant encouragement, and excellent curries!

Contents

List of Figures

List of Tables

List of Abbreviations

3α-HSD, 3β-HSD	3α-hydroxy-steroid dehydrogenase, 3β-hydroxy-steroid dehydrogenase
aCSF	Artificial cerebrospinal fluid
AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ANOVA	Analysis of variance
AP2	Clathrin adaptor protein 2
ASPA	Animals (Scientific Procedures) Act, 1986
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
CA1 – CA3	*Cornu ammonis* (CA) regions 1-3 of the hippocampus
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
cDNA	Complementary deoxy-ribonucleic acid
CNS	Central nervous system
CSF	Cerebrospinal fluid
d.f.	Degrees of freedom
DG	Dentate gyrus
DHEA	Dehydro-epi-androsterone
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulphoxide
EC_{15}	The concentration of substance producing a response 15% of the maximal
EC_{50}	The concentration of substance producing a response 50% of the maximal
EDTA	Ethylene-diamine-tetra-acetic acid
EEG	Electro-encephalogram
eGFP	Enhanced green fluorescent protein
ES cell	Embryonic stem cell
GABA	Gamma amino butyric acid
$GABA_A$, $GABA_B$, $GABA_C$	GABA receptor subclasses A-C
GABARAP	$GABA_A$-receptor-associated protein
GAD65, GAD67	Glutamate decarboxylases 65 and 67
GC	Granule cell
HAP-1	Huntingtin-associated protein 1
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Het	Heterozygous, heterozygote
Hom	Homozygous, homozygote
HPA axis	Hypothalamic-pituitary-adrenal axis
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
I.E.I.	Inter-event interval
I.F.	Immunofluorescence
ICSS	Intra-cranial self-stimulation
IPSC	Inhibitory postsynaptic current
kb	Kilo-base-pairs
kDa	Kilo-Daltons
KIF5	Kinesin family motor protein 5
LSD	Least significant difference

Lux	Light intensity measure, equivalent to lumens per square metre
M1 – M4	Transmembrane helices 1 - 4
MF	Mass fragmentography
mIPSC	Miniature inhibitory postsynaptic current
mRNA	Messenger ribonucleic acid
MSN	Medium spiny neuron
NA	Numerical aperture
NAcc	Nucleus Accumbens
NMDA	N-Methyl-D-aspartate
P450c17	17α hydroxylase, c17,20 lyase
P450scc	Cytochrome P450 cholesterol side-chain cleavage
PAGE	Poly-acrylamide gel electrophoresis
PB	Phosphate buffer
PBS	Phosphate buffered saline
PC	Pyramidal cell
PCR	Polymerase Chain Reaction
PKA, PKB, PKC, PKG	Protein kinases A, B, C and G
PLIC1	Protein that links integrin-associated protein with the cytoskeleton-1
PP1, PP2A	Protein phosphatases 1 and 2A
PRIP	Phospholipase-C related inactive protein
Pxx (e.g. P18)	Postnatal age xx days
r.m.s.	Root mean square
r.p.m.	Rotations per minute
RACK1	Receptor for activated C kinase1
Raft1	Rapamycin and FKB12 target protein
REM sleep	Rapid eye movement sleep
ROI	Region of interest
Rs	Series resistance
RT	Room temperature
s.e.m.	Standard error of the mean
SDS	Sodium-dodecyl sulphate
sIPSC	Spontaneous inhibitory postsynaptic current
SON	Supraoptic nucleus
SPECT	Single photon emission computed tomography
SSRI	Selective serotonin reuptake inhibitor
StAR	Steroidogenic acute regulatory protein
THDOC	Allo-tetrahydro-deoxy-corticosterone (3α,21-dihydroxy-5α-pregnan-20-one)
TSPO	18 kDa translocator protein, also called ‘peripheral benzodiazepine receptor’
Veh	Vehicle
VTA	Ventral tegmental area
WB	Western blot
Wt	Wild-type
γ2L, γ2S	Long (L) and short (S) isoforms of the γ2 subunit

Chapter 1: Introduction

Neurosteroid modulation of GABAergic neurotransmission in physiology and pathophysiology

1.1. $GABA_A$ receptors

Gamma-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS), playing a central role in regulating neuronal excitability. GABA fulfils this role by activating two classes of receptor: ionotropic type-A receptors ($GABA_A$ receptors) and metabotropic type-B ($GABA_B$) receptors. The focus of this study is the $GABA_A$ receptor family, which encompasses Cl^- and bicarbonate-permeable channels of the Cys-loop-containing ligand-gated ion channel family (of which the nicotinic acetylcholine receptor is the founding member) (Barnard *et al.*, 1998), now known as the pentameric ligand-gated ion channels (Miller & Smart, 2010; Corringer *et al.*, 2012).

Excitatory and inhibitory neurotransmission are finely balanced processes, and any disruption of this balance can be detrimental to brain function. Improper GABAergic signalling is seen in neurodegenerative diseases such as Huntington's, after ischemic episodes, and also in epilepsy, anxiety disorders, depression and schizophrenia (Fritschy & Brunig, 2003; Mohler, 2006b). It is therefore unsurprising that $GABA_A$ receptors represent a major pharmacological target for the treatment of these disorders. This thesis will focus on the roles of $GABA_A$ receptors in anxiety and depression, with particular attention paid to neurosteroids as players in the disease process, as well as assessing their potential as therapeutic agents for these disorders.

1.1.1. $GABA_A$ receptor composition and function

The $GABA_A$-receptor gene family encompasses several subunits, some of which are present in multiple isoforms (α1-6, β1-3, γ1-3, δ, ε, π and ρ1-3 (Barnard *et al.*, 1998; Korpi *et al.*, 2002)), with further diversity imparted by alternative splicing (e.g. γ2 is expressed as short and long splice forms – γ2S and γ2L, respectively (Whiting *et al.*, 1990; Kofuji *et al.*, 1991; Glencorse *et al.*, 1992)). When studied in recombinant expression systems, functional receptors may assemble from a single subunit (e.g. β or ρ), or two subunits (e.g. α with β). However, the majority of native receptors *in vivo* are thought to be pentamers of 2α, 2β and 1x subunit (*Fig. 1.1*), where x is typically a γ subunit in synaptic receptors, but could be δ in receptors outside the synapse (Brickley *et al.*, 1999; Mody, 2001; Moss & Smart, 2001; Sieghart & Sperk, 2002; Mohler, 2006a). These rules are not strict, however, since typically synaptic subunits are found outside of synapses too (e.g. α1 and γ2 in electron micrographs of cerebellar granule cells (Nusser *et al.*, 1998)), and αβ receptors (i.e. without γ or δ subunits) have been observed in hippocampal neurons (Mortensen & Smart, 2006). The γ subunit in αβγ receptors may be replaced by ε or π, whilst θ subunits are thought to take the place of β subunits (Sieghart & Sperk, 2002). Expression of subunits ρ1-3 is mostly restricted to the retina, where they exist as homo- or hetero-oligomers with properties distinct from αβx assemblies, leading to their separate classification as $GABA_C$ receptors (Sieghart & Sperk, 2002).

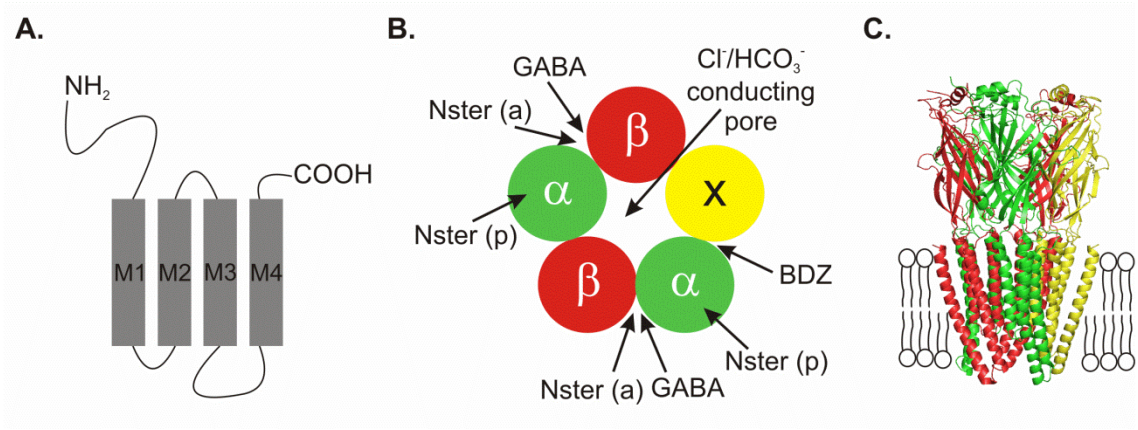

Figure 1.1 – GABA$_A$ receptor subunit assembly and modulator binding sites

Each GABA$_A$ receptor subunit has the depicted topology (A) of a large extracellular amino-terminal (NH_2) domain, four transmembrane helices (M1-4), and a short extracellular carboxy-terminus (COOH) (Korpi *et al.*, 2002). The large intracellular loop between M3 and M4 helices is a key site for protein-protein interactions and phosphorylation, both of which are means for regulating receptor location and function (see main text). Subunits are thought to assemble as pentamers of stoichiometry 2α:2β:1x subunits (Chang *et al.*, 1996; Tretter *et al.*, 1997; Farrar *et al.*, 1999). The proposed arrangement of subunits is depicted as viewed from the extracellular surface (B) or in the plane of the membrane (C). The locations of binding sites for GABA, benzodiazepines (BDZ, note that this site depends on x being a γ subunit) and neurosteroid activation (Nster (a)) and neurosteroid potentiation (Nster (p)) are indicated (Korpi *et al.*, 2002; Hosie *et al.*, 2006; Hosie *et al.*, 2009).

Given the large number of subunit isoforms, there are theoretically many permutations of αβx assemblies. However, far fewer combinations are actually found *in vivo*, depending on which subunits are co-expressed within a neuron (McKernan & Whiting, 1996; Sieghart & Sperk, 2002; Mohler, 2006a). Distinct expression profiles are observed for each subunit across brain areas, which alter with development (Laurie *et al.*, 1992a; Laurie *et al.*, 1992b; Wisden *et al.*, 1992; Pirker *et al.*, 2000). Furthermore, within a particular brain region and/or cell type, there can be cell-to-cell variations in receptor expression (e.g. using single-cell polymerase chain reaction (PCR) measurements of messenger ribonucleic acid (mRNA) levels, some cerebellar granule cells appear to express only α1, others only α6, and a third subset express both (Santi *et al.*, 1994)). The likely receptor subunit combinations formed *in vivo* have been

defined by examining protein co-distribution with immunostaining and mRNA co-distribution by *in situ* hybridisation (Somogyi *et al.*, 1989; Fritschy *et al.*, 1992; Wisden & Seeburg, 1992), and co-immunoprecipitation of subunits from brain tissue (Benke *et al.*, 1991; McKernan & Whiting, 1996). The two most abundant receptor combinations in rat brain are thought to be α1β2γ2 (40-60%) and α2β2/3γ2 (15-20%); other less common combinations include α3βnγ2 (10-15%), α4βnγ2/α4βnδ (<5%), α5βnγ2 (<5%) and α6βnγ2/α6βnδ (<5%) (McKernan & Whiting, 1996; Sieghart & Sperk, 2002; Mohler, 2006a). It is also possible that the two copies of α or β subunits within a receptor can be different isoforms – e.g. α1α3β2/3γ2 receptors (Fritschy *et al.*, 1992).

The agonist-gated pore of $GABA_A$ receptors allows transmembrane passage of Cl^- and HCO_3^- ions; the direction of the ion flow is determined by their transmembrane electrochemical gradients, which can be developmentally regulated (switching from excitatory to inhibitory during brain development (Ben-Ari, 2002)). Depending on their properties and subcellular location, $GABA_A$ receptors can carry two types of current: 'phasic' and 'tonic' (Mody, 2001; Semyanov *et al.*, 2004; Farrant & Nusser, 2005). Phasic events, or inhibitory post synaptic currents (IPSCs), involve action-potential stimulated release of GABA at the synaptic cleft, which stimulates opening of synaptically-located $GABA_A$ receptors; events are short-lived, due to rapid GABA clearance from the cleft. In contrast, tonic currents result from more persistent activation of $GABA_A$ receptors, which are responding to the low ambient GABA levels outside the synapse (Mody, 2001; Semyanov *et al.*, 2004; Farrant & Nusser, 2005). Both types of inhibition can be considered a means to counter excessive neuronal network excitation; phasic currents are also involved in generating rhythmic network activities, whilst tonic inhibition can modulate the input-output relationship for excitatory signalling onto a particular cell (Semyanov *et al.*, 2004; Farrant & Nusser, 2005).

The source of ambient GABA responsible for tonic currents varies, but can involve spillover of synaptically released GABA, reverse transport at synapses and non-synaptic sources of GABA (including release from astrocytes)

(Richerson, 2004; Semyanov *et al.*, 2004; Farrant & Nusser, 2005). The importance of vesicular release is supported by the reduction in extracellular GABA concentrations after blocking action potentials with tetrodotoxin (Bianchi *et al.*, 2003; Xi *et al.*, 2003) and also by the strong positive correlation between phasic and tonic current amplitudes recorded from brain slices under a range of conditions (Glykys & Mody, 2007). Nevertheless, the residual measures of extracellular GABA after tetrodotoxin treatment of the hippocampus (Bianchi *et al.*, 2003) or nucleus accumbens (Xi *et al.*, 2003) indicate that as much as 75% of extracellular GABA in these regions is of non-vesicular origin.

In order to pass current during prolonged exposure to low ambient GABA concentrations, tonic-carrying receptors must have a higher GABA affinity and slower desensitisation kinetics than those involved in phasic currents (Farrant & Nusser, 2005). Approaches to determine the identity of tonic vs. phasic receptors include imaging (to demonstrate appropriate subcellular localisation), pharmacology (demonstrating current sensitivity to subunit-selective compounds) and subunit knock-outs (loss of current when subunit x is ablated). The results of these experiments are not always unequivocal, probably because the identity of receptors passing each type of current varies across cell types, and within a particular cell type according to the recording conditions employed. This is perhaps best illustrated by considering the reports for tonic currents in hippocampal pyramidal cells (PCs), particularly those in the *cornu ammonis* 1 (CA1) region. Semyanov *et al.* (2003) found no detectable tonic current in PCs from guinea pig hippocampi at baseline, and required inhibition of GABA uptake to reveal a tonic current. In contrast, Prenosil *et al.* (2006) observe a clear tonic current in CA1 PCs without manipulating extracellular GABA levels. Where a tonic current has been detected in wild-type CA1 PCs, pharmacological profiling pointed to roles for α5, β2/3 and γ2, whilst suggesting no involvement of α4, α6, ε or δ subunits (Caraiscos *et al.*, 2004). In addition, knock-out of α5 subunits diminished tonic current recorded from CA1 (Caraiscos *et al.*, 2004). However, a role for α5 subunits is not universally supported: for example, Prenosil *et al.* (2006) find their tonic current to be insensitive to L655-708, an α5-selective benzodiazepine-site inverse agonist. A role for δ subunits in tonic currents seemed unlikely on the basis of several observations: δ subunit knock-out mice

(δ-/- mice) have undiminished tonic currents in hippocampal pyramidal cells in culture (Caraiscos *et al.*, 2004) and brain slice tissue (Stell *et al.*, 2003; Glykys *et al.*, 2008), and the tonic current of wild-type cells is insensitive to the neurosteroid THDOC (allo-tetrahydro-deoxy-corticosterone) (Stell *et al.*, 2003). However, they cannot rule out compensatory alterations in response to losing the δ subunit, nor does THDOC insensitivity necessarily imply a lack of δ subunit contribution. Indeed, a role for δ subunits has more recently been acknowledged, because the residual tonic current in CA1 PCs of α5-/- mice is lost in double knockout α5-/-δ-/- mice (Glykys *et al.*, 2008). Interestingly, treatment of slices from δ-/- mice with L-655,708 does not abolish the tonic current (Glykys *et al.*, 2008), suggesting yet more subunits could contribute to tonic currents in CA1 PCs. Indeed, there is evidence for a contribution of αnβn combinations to tonic currents of cultured hippocampal pyramidal cells (Mortensen & Smart, 2006). Data therefore predict a dominant role for α5β2/3γ2 receptors in determining CA1 PC tonic current, but do not rule out contributions from other subunit combinations. On the basis of results from similar experimental approaches, cerebellar granule cell tonic current is believed to be mostly mediated by α6βnδ receptors (Brickley *et al.*, 2001), and that in dentate gyrus granule cells (DG GC) by α4βnδ (Nusser & Mody, 2002; Stell *et al.*, 2003).

Regardless of the identity of receptors passing GABAergic currents, it is appreciated that this neurotransmission is highly plastic, and is modulated by a number of physiological and pharmacological processes (Luscher & Keller, 2004). Mechanisms exist to alter the density of receptors in a given membrane region, as well as the amount of current that flows through these receptors in response to GABA binding. Some of these mechanisms are described in the following sections (*1.1.2 and 1.1.3*).

1.1.2. $GABA_A$ receptor modulation: trafficking

The regulation of synaptic $GABA_A$ receptor trafficking and the roles of its many receptor-associated partners in these processes has been extensively reviewed elsewhere (Kittler & Moss, 2001; Moss & Smart, 2001; Fritschy & Brunig, 2003; Kittler & Moss, 2003; Luscher & Keller, 2004; Arancibia-Carcamo & Kittler, 2009; Luscher *et al.*, 2011b). A full discussion of these mechanisms is beyond the scope of this thesis, but some of the key components (shown in *Fig. 1.2*) will be described below. The majority of the $GABA_A$ receptor binding partners associate with the intracellular domain between transmembrane helices M3 and M4, and so it is unlikely that all of these interactions will take place at once. Complexes are likely to be dynamic and transient in nature, allowing regulation of receptor actions at particular locations and under specific conditions.

Gephyrin and γ2 subunits appear to be interdependent for synaptic clustering of $GABA_A$ receptors (Essrich *et al.*, 1998; Fischer *et al.*, 2000), although the interaction with gephyrin occurs via α subunits (Tretter *et al.*, 2008; Tretter *et al.*, 2011). There are also gephyrin-independent means of receptor clustering, such as interactions with dystrophin (Knuesel *et al.*, 1999). Gephyrin's roles probably extend beyond synaptic anchoring of $GABA_A$ and glycine receptors, because it interacts with numerous binding partners that may regulate cytoskeleton dynamics, local protein translation and receptor trafficking (*Fig. 1.2*).

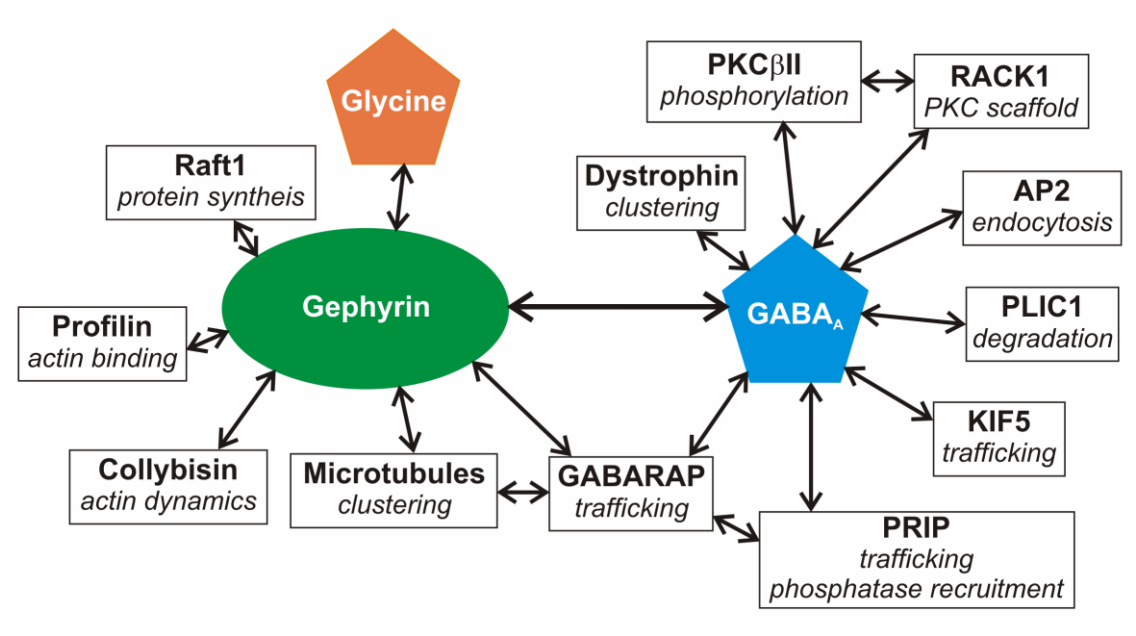

Figure 1.2 –GABA$_A$ receptor binding partners

GABA$_A$ receptors are modulated by interactions with a host of other proteins, some of which are discussed in the main text. Receptor delivery to the cell surface involves trafficking proteins GABARAP (GABA$_A$ receptor associated protein), KIF5 (kinesin family motor protein 5) and PRIP (phospholipase-C related inactive protein), whilst endocytosis for recycling or degradation involves proteins AP2 (clathrin adaptor protein 2) and PLIC1 (protein that links integrin-associated protein with the cytoskeleton-1). Synaptic clustering of receptors involves interaction with dystrophin or with gephyrin, which itself interacts with a number of proteins. Through these interactions, gephyrin may bind to and regulate the cytoskeleton, as well as modulate protein translation via Raft1 (rapamycin and FKB12 target protein). GABA$_A$ receptors can also bind proteins involved in modulating receptor phosphorylation state, such as RACK1 (receptor for activated C kinase1), PKCβII (protein kinase C, isoform βII) and PRIP. Phosphorylation serves to regulate GABA$_A$ receptor function and trafficking, and may do so by modulating receptor interactions with its binding partners (see main text).

Receptor targeting mechanisms are more sophisticated than simply directing to a synaptic or extrasynaptic site: specific receptors can be localised at particular synapses within a neuron. For example, within hippocampal pyramidal cells, α1 subunits are uniformly distributed, but α2 subunits are concentrated at synapses local to the axon initial segment (Nusser *et al.*, 1996). Targeting may depend on signals from the presynaptic cell, because distinct interneurons synapse onto

these different regions of the principal cell: α1 subunits are at synapses with parvalbumin-positive basket cells, whilst α2 subunits are clustered at synapses from cholecystokinin-positive basket cells and parvalbumin-positive chandelier cells (Nyiri *et al.*, 2001; Luscher & Keller, 2004; Mohler, 2006a)). Subcellular targeting has been probed using an artificial synapse model, which demonstrates that as-yet undefined determinants within α2 vs. α6 subunits direct them to the appropriate location (Wu *et al.*, 2012). Indeed, ectopically-over-expressed α6 subunits can direct receptors to extrasynaptic sites within neurons, and 'dominates' over γ2 subunits within the same receptor in this targeting (Wisden *et al.*, 2002).

Although anchored by gephyrin and/or dystrophin, synaptic $GABA_A$ receptors are by no means static entities. Receptors are constantly trafficking into and out of the synaptic zone by membraneous transport (exo- and endocytosis), as well as by lateral mobility within the membrane. Receptor internalisation involves interaction of β and/or γ subunits with the clathrin adaptor protein, AP2, depending on their phosphorylation state (Kittler *et al.*, 2000; Kittler *et al.*, 2005; Luscher *et al.*, 2011a). After internalisation, receptors may either recycle back to the cell surface, or are targeted to the lysosome for degradation. The latter route is favoured by lysine ubiquitination of the γ subunit (Arancibia-Carcamo *et al.*, 2009), whilst cell surface re-delivery is favoured by interaction of PLIC1 with α and β subunits (Bedford *et al.*, 2001). Cell surface delivery of $GABA_A$ receptors is also facilitated by interaction of β subunits with PRIP (Kanematsu *et al.*, 2006; Mizokami *et al.*, 2007) and the kinesin family motor protein, KIF5 (via adaptor protein Huntingtin-associated protein 1, HAP1 (Twelvetrees *et al.*, 2010)). Trafficking from the Golgi apparatus to the cell surface also involves interaction of γ subunits with GABARAP and with tubulin (the latter interaction may occur via GABARAP and/or through HAP1-KIF5 complexes) (Item & Sieghart, 1994; Wang *et al.*, 1999; Kneussel *et al.*, 2000; Kittler *et al.*, 2001). The cycling of $GABA_A$ receptors into and out of the cell surface membrane appears to be occurring constitutively, because disruption of either process can influence both the cell surface expression of γ-subunit containing $GABA_A$ receptors, and synaptic GABAergic currents. For example, over a short timescale (minutes) disruption of clathrin-mediated endocytosis increases

synaptic current amplitudes (Kittler *et al.*, 2000); conversely, impairing cell surface delivery by disrupting the KIF5-HAP1 interaction reduces current amplitudes (Twelvetrees *et al.*, 2010). Furthermore, despite their interaction with anchoring proteins, synaptic $GABA_A$ receptors also show lateral mobility within the membrane (Thomas *et al.*, 2005). Movement of receptors into and out of synaptic sites by this mechanism may represent the major means of altering synaptic strength, and may occur over faster timescales than vesicular transport mechanisms (Thomas *et al.*, 2005).

1.1.3. $GABA_A$ receptor modulation: ligands and post-translational modification

A number of endogenous and exogenous ligands are allosteric modulators or direct agonists of $GABA_A$ receptors including barbiturates, benzodiazepines, neurosteroids and Zn^{2+} (details below). $GABA_A$ receptor activity can also be modulated by direct modifications of the channel, including phosphorylation (details below), protonation (Huang & Dillon, 1999; Wilkins *et al.*, 2002, 2005) and redox modifications of cysteine residues (Amato *et al.*, 1999; Pan *et al.*, 2000).

Pharmacological modulation by barbiturates and benzodiazepines

The effects of barbiturates on $GABA_A$ receptor currents depends on their concentration: potentiation, direct activation and inhibition occur with increasing concentration (e.g. pentobarbitone shows all three responses, with efficacies dependent on receptor subunit composition (Thompson *et al.*, 1996)). By increasing $GABA_A$ receptor currents, these compounds are effective as anxiolytics and hypnotics. However, clinical barbiturate use has now been superseded by that of benzodiazepines, which are also allosteric potentiators of $GABA_A$ receptors, but are safer in overdose (Smith & Rudolph, 2012).

Responses to classical benzodiazepines (e.g. diazepam) require the presence of a γ subunit and absence of the benzodiazepine-insensitive α4 or α6 isoforms

(Pritchett *et al.*, 1989; Wieland *et al.*, 1992). Their numerous behavioural effects have been linked to their action at distinct $GABA_A$ receptor α subunit isoforms (*Table 1.1*). Targeting benzodiazepines to specific α subunits may therefore produce specific behavioural effects. Particular efforts have focussed on generating α2-targeting benzodiazepines as non-sedating anxiolytics (see *Section 1.3.2*).

Isoform	Benzodiazepine effect	References
α1	sedation, amnesia, anticonvulsion	(Rudolph *et al.*, 1999; McKernan *et al.*, 2000)
α2	anxiolysis and myorelaxation	(Low *et al.*, 2000; Crestani *et al.*, 2001)
α3	anxiolysis[1] and myorelaxation	(Crestani *et al.*, 2001; Dias *et al.*, 2005)
α5	sedative tolerance	(van Rijnsoever *et al.*, 2004)

Table 1.1 – Subunit-specific actions of benzodiazepines

There are six α subunit isoforms, and whilst all are neurosteroid sensitive (Hosie *et al.*, 2009), α4 and α6 subunits preclude benzodiazepine response due to a single amino acid substitution (H to R) in the otherwise conserved binding site (Wieland *et al.*, 1992). Introducing this substitution into the benzodiazepine-responsive subunits (i.e. $\alpha1^{H101R}$, $\alpha2^{H101R}$, $\alpha3^{H126R}$ or $\alpha5^{H105R}$) abolishes their response to benzodiazepines in recombinant systems, but leaves other properties of the channel unaffected (Wieland *et al.*, 1992; Benson *et al.*, 1998). This table summarises the results of characterising knock-in mice with each of these point mutations, which allowed dissection of the isoforms responsible for the various behavioural effects of benzodiazepines.

[1]Note that $\alpha3^{H126R}$ mice did not lack anxiolytic response to diazepam (Low *et al.*, 2000), but a role for this subunit was supported by observations of anxiolysis with an α3-selective benzodiazepine (Dias *et al.*, 2005)

Endogenous modulation by Zn^{2+}

Experimentally, transmission through $GABA_A$ receptors can be modulated by Zn^{2+} ions either directly (inhibitory effects on subunit combinations lacking γ2 subunits) or indirectly (by enhancing synaptic release of GABA in the

hippocampus) (Hosie *et al.*, 2003; Smart *et al.*, 2004). Histological stains for Zn^{2+} demonstrate a strong presence in the telencephalon, hippocampus, cerebral cortex and amygdala, suggesting that Zn^{2+} modulation occurs endogenously (Smart *et al.*, 2004). Chelation of Zn^{2+} ions increased the amplitude of CA3 pyramidal cell IPSCs evoked by stimulation of mossy fiber inputs, confirming a role for baseline endogenous Zn^{2+} in modulating GABAergic transmission (Ruiz *et al.*, 2004).

Endogenous modulation by kinases/phosphatases

The intracellular loops of β and γ subunits are phosphorylated at a number of residues (*Table 1.2*) found within consensus sequences for a number of protein kinases. Phosphorylation at these sites may increase or decrease GABAergic currents by modulating receptor gating properties and/or trafficking processes that determine the number of receptors at the cell surface (see reviews: Smart, 1997; Brandon *et al.*, 2002; Kittler & Moss, 2003; Song & Messing, 2005; Arancibia-Carcamo & Kittler, 2009; Houston *et al.*, 2009b). The precise effects on GABAergic currents depend not only on the kinase and subunit isoforms in question, but also seem to vary with the preparation being studied. For example, Ca^{2+}/calmodulin-dependent kinase II (CaMKII) failed to modulate receptors expressed in human embryonic kidney 293 (HEK293) cells, but did so if receptors were expressed in a neuroblastoma cell line (Houston *et al.*, 2009b).

Protein kinase C (PKC)-dependent phosphorylation can increase cell surface expression of receptors by disrupting the interaction between β/γ subunits and AP2 (Kittler *et al.*, 2000; Kittler *et al.*, 2005; Luscher *et al.*, 2011a). Interestingly, protein kinase B (PKB/Akt)-dependent phosphorylation of the same residues as PKC, rather than influencing receptor internalisation, increases cell surface delivery of newly-synthesised receptors – i.e. phosphorylation effects depend not only on the residues acted upon, but also where in the cell it takes place (Luscher *et al.*, 2011a). It must not be forgotten that $GABA_A$ receptors are not the only target of these enzymes: Connolly *et al.* (1999) found that stimulating PKC in HEK293 cells decreased cell surface expression of α1β2γ2 receptors, but that the effect does not require phosphorylation at $\beta2^{S410}$, $\gamma2^{S327}$ or $\gamma2^{S343}$.

$GABA_A$ receptor subunit	Residues	Kinases
β1	S409	PKA[1], PKB[5], PKC[1,3], PKG[1]
	S384, S409	CaMKII[4]
β2	S410	PKA[1], PKB[2,5], PKC[1,3], PKG[1], CaMKII[1,4]
β3	S408, S409	PKA[1], PKB[5], PKC[1,3], PKG[1]
	S383, S409	CaMKII[4]
γ2	S327, <u>S343</u>	PKC[1,3], CaMKII[1,4]
	S348, T350	CaMKII[4]
	Y365, Y367	Tyrosine kinases[1,2]

Table 1.2 – Phosphorylation sites on $GABA_A$ receptors

$GABA_A$ receptor β and γ subunit phosphorylation sites are listed alongside the kinases that are able to phosphorylate them. Underlined residues are unique to the γ2L isoform (i.e. absent from the γ2S isoform). Table compiled using reviews by [1]Brandon *et al.* (2002), [2]Luscher and Keller (2004), [3]Song and Messing (2005), [4]Houston *et al.* (2009b), [5]Luscher *et al.* (2011a).

Phosphorylation is a readily-reversible post-translational modification, and so receptor phosphorylation state is likely to be dynamically regulated *in vivo*. Indeed, brain-derived neurotrophic factor (BDNF) stimulation of cultured hippocampal and cortical neurons induces a transient rise and fall in β3 subunit phosphorylation (Jovanovic *et al.*, 2004; Kanematsu *et al.*, 2006). Synaptic current amplitudes increase in parallel with the PKC-mediated β3 phosphorylation, which is temporally associated with increased cell surface expression of receptors (Jovanovic *et al.*, 2004). The latter stages of the BDNF response, where synaptic current amplitudes diminish and β3 subunits are dephosphorylated, involve the recruitment of protein phosphatases 1 and 2A (PP1/PP2A) by PRIP (Kanematsu *et al.*, 2006). It is not clear whether this latter stage involves reduced cell surface expression (Kanematsu *et al.*, 2006), or reduced currents without an altered surface expression (Jovanovic *et al.*, 2004). Nevertheless, these investigations concur that β3 phosphorylation is only transient in nature after BDNF treatment.

Neurosteroids as endogenous modulators

Steroid compounds not only modulate gene expression by binding to transcription factors of the nuclear receptor family, but they also have non-genomic actions within the CNS (Paul & Purdy, 1992; Rupprecht & Holsboer, 1999; Belelli *et al.*, 2006). These “neuroactive steroids”, or “neurosteroids”, are compounds that are produced endogenously and can affect the function of several neuronal receptors, including NMDA (*N*-Methyl-D-aspartate), AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid), kainate, glycine, serotonin, nicotinic acetylcholine, oxytocin, sigma-type-1 and $GABA_A$ receptors (Rupprecht & Holsboer, 1999; Mellon & Griffin, 2002; Strous *et al.*, 2006). The two major neurosteroids *in vivo*, allopregnanolone and allo-tetrahydro-deoxy-corticosterone (THDOC), are among the most potent known endogenous modulators of $GABA_A$ receptors (Paul & Purdy, 1992). Immunohistochemical analysis demonstrates that these two neurosteroids are mostly concentrated in cell bodies and dendrites of excitatory neurons, with little or no labelling of gliaform cells or inhibitory interneurons, suggesting that they act in a paracrine or autocrine manner to modulate principal cell firing (Saalmann *et al.*, 2007).

That $GABA_A$ receptors are a major molecular target for neurosteroids was made apparent by a combination of radioisotope (measuring $^{36}Cl^-$ flux into synaptosomes) and electrophysiological studies. These studies demonstrated that the action of neurosteroids and their analogues is biphasic: low (nM) concentrations augment GABA-induced Cl^- conductance, whereas higher (µM) concentrations directly stimulate $GABA_A$ receptor activation – producing slow inward currents that resemble the direct responses to pentobarbital (Harrison & Simmonds, 1984; Harrison *et al.*, 1987; Puia *et al.*, 1990; Paul & Purdy, 1992). The majority of estimates for *in vivo* neurosteroid concentrations support a role for potentiation at $GABA_A$ receptors, but sufficient levels to directly activate these receptors may be achieved under specific circumstances, such as in mother and foetus during late pregnancy (Ichikawa *et al.*, 1974; Paul & Purdy, 1992; Biedermann & Schoch, 1995; Luisi *et al.*, 2000; Nguyen *et al.*, 2003).

At the molecular level, neurosteroids are thought to act by altering the kinetics of receptor entry into and exit from desensitised states (Zhu & Vicini, 1997) and may increase the efficacy of ion channel gating (Bianchi & Macdonald, 2003). At the cellular level, neurosteroid-mediated potentiation at $GABA_A$ receptors enhances synaptic (Belelli & Herd, 2003; Harney *et al.*, 2003) and tonic currents (Stell *et al.*, 2003). Both responses are expected to hyperpolarize the cell membrane and/or shunt excitatory inputs, and thus reduce the neuron's probability of firing. There is also a class of neurosteroids that antagonize $GABA_A$ function: the so-called "excitatory steroids" – mostly sulphated forms of the classical neurosteroids, such as pregnenolone sulphate, or 3β-hydroxy-steroids (Akk *et al.*, 2001; Wang *et al.*, 2002). They are pro-convulsant in animals, act as non-competitive antagonists at the $GABA_A$ receptor, and probably bind to a site distinct from potentiating neurosteroids; their effects in animals may also be due to an enhancement of excitatory glutamatergic transmission (Paul & Purdy, 1992; Akk *et al.*, 2001; Wang *et al.*, 2002; Hosie *et al.*, 2007).

In our study, we hope to increase understanding of the roles played by endogenous neurosteroids, specifically via positive allosteric modulation at α2-type $GABA_A$ receptors. We have particularly focused on their roles in the aetio-pathology of anxiety and depression. Furthermore, we have explored therapeutic potential for α2-subunit-targeting neurosteroids in these disorders. The following sections therefore provide details regarding current knowledge of neurosteroid physiology (*Section 1.2*) and its links to anxiety (*Section 1.3*) and depression (*Section 1.4*).

1.2. Neurosteroid physiology and pharmacology

1.2.1. Endogenous neurosteroids: synthesis and roles

Neurosteroids are synthesized *in vivo* from a cholesterol precursor, via a range of enzymatic conversions (see *Fig. 1.3*). Neurosteroid distribution within the brain is not uniform, with the highest levels generally in the olfactory bulb, striatum and cortex, and lower levels in brainstem, suggesting there will be a regional variation in neurosteroidogenesis and neurosteroid-mediated modulation of GABAergic transmission (Uzunov *et al.*, 1996; Bixo *et al.*, 1997; Bernardi *et al.*, 1998; Saalmann *et al.*, 2007). Neurosteroids and their precursors can also be produced in peripheral steroidogenic tissues such as the adrenal cortex and gonads. For example, menstrual-cycle-related changes in circulating progesterone can influence brain allopregnanolone levels, with key consequences for sufferers of catamenial epilepsy (see *Section 1.2.4*). Furthermore, stress-induced rises in brain neurosteroid levels are thought to involve production of these compounds in the adrenal glands (Purdy *et al.*, 1991; Barbaccia *et al.*, 1998). However, a change in peripheral levels cannot necessarily be projected to cause changes in CNS levels of a given neurosteroid. Indeed, the rises in serum and brain allopregnanolone and THDOC levels during pregnancy do not directly correlate with the rise in serum progesterone (in rats (Concas *et al.*, 1998) or humans (Luisi *et al.*, 2000)) – i.e. peripheral progesterone is not the only source for brain allopregnanolone.

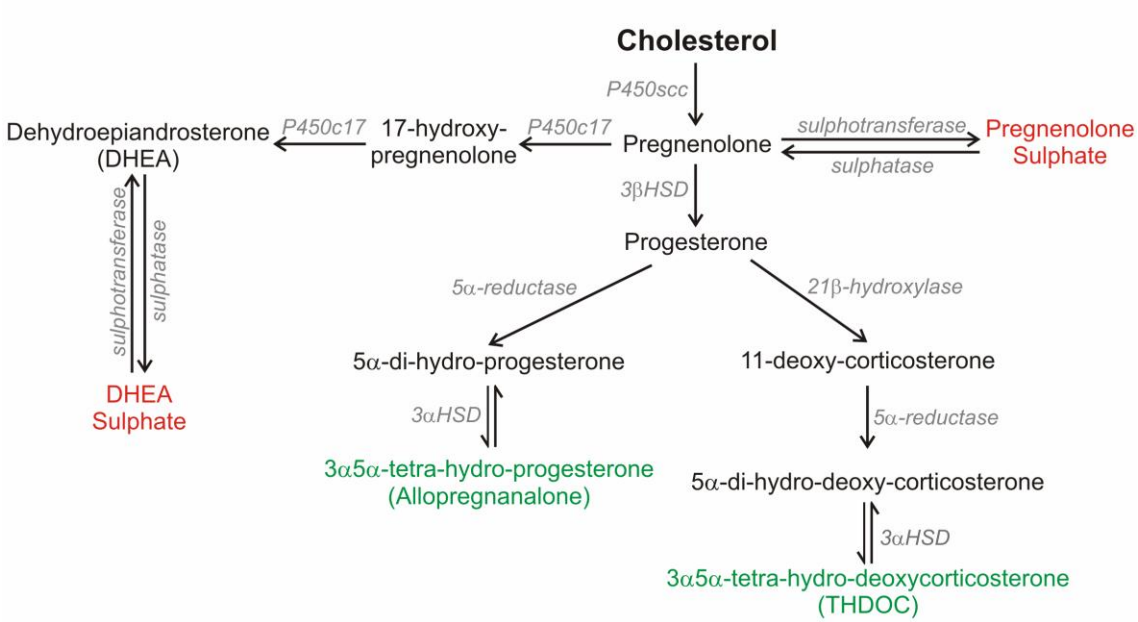

Figure 1.3 – Neurosteroid synthesis pathways

The steps involved in converting cholesterol into $GABA_A$ receptor modulating neurosteroids are shown. A number of additional neuroactive compounds can also be produced by modifications of the various intermediates (black) (for details, see Mellon & Griffin, 2002; Stoffel-Wagner, 2003), but this figure focuses on the major positive (green) and negative (red) allosteric modulators of $GABA_A$ receptors. The neurosteroid profile of a particular brain region will depend on the enzymes present locally; in humans, hippocampal and temporal lobe expression and/or enzymatic activity has been demonstrated for P450scc, 21β-hydroxylase, 5α-reductase and 3α-HSD (Stoffel-Wagner, 2003). The initial conversion of cholesterol to pregnenolone occurs within mitochondria, requiring the activity of two transporters: StAR (steroidogenic acute regulatory protein (Stocco & Clark, 1996)) and TSPO (the 18 kDa translocator protein), the latter of which is sensitive to stimulation by benzodiazepines (Papadopoulos & Lecanu, 2009). Abbreviations: 3β-HSD, 3β-hydroxy-steroid dehydrogenase; 3α-HSD, 3α-hydroxy-steroid dehydrogenase; P450scc, cytochrome P450 cholesterol side-chain cleavage; P450c17, 17α hydroxylase, c17,20 lyase. Note that the HSD enzymes are also referred to as hydroxy-steroid oxido-reductase (HSOR) enzymes.

The levels of neurosteroids have been measured most commonly by radio-immunoassay (Purdy *et al.*, 1990a) or by mass fragmentography (MF) (Uzunov *et al.*, 1996). The former method is limited by the antibody specificity, but pre-separation of the various steroids extracted from the sample by high-

performance liquid chromatography (HPLC) can eliminate the problem of cross-reactivity with other neurosteroid metabolites (Purdy *et al.*, 1990a). The MF approach is more technically demanding, but has been proposed to be a more sensitive approach, capable of accurately measuring picomolar levels of steroid (Uzunov *et al.*, 1996). The estimates of baseline rodent brain neurosteroid levels vary, with reports for allopregnanolone ranging from very low (<3 nM (Purdy *et al.*, 1991; Vallee *et al.*, 2000)), to levels sufficient to potentiate $GABA_A$ receptors (3-10 nM (Uzunov *et al.*, 1996); 2-20 nM (Bernardi *et al.*, 1998)). Baseline allopregnanolone levels in human brains may be higher (e.g. in women, may range from 30-70 nM, depending on serum progesterone levels (Bixo *et al.*, 1997)), but human brain samples are less readily available, and obtained post-mortem.

Allopregnanolone and THDOC levels increase during pregnancy (Concas *et al.*, 1998; Luisi *et al.*, 2000) and in response to stressors (Purdy *et al.*, 1991; Paul & Purdy, 1992) or drugs – including nicotine (Porcu *et al.*, 2003), gamma-hydroxy butyrate (Barbaccia *et al.*, 2002) and some antidepressants (Uzunov *et al.*, 1996). Increased production of endogenous neurosteroids is also believed to mediate some of the behavioural responses to ethanol, including its antidepressant and anticonvulsant effects (VanDoren *et al.*, 2000; Hirani *et al.*, 2002; Khisti *et al.*, 2002; Helms *et al.*, 2012).

Dysregulation of neurosteroids is associated with a range of diseases, including premenstrual dysphoric disorder, panic disorder, anxiety and stress, depression, schizophrenia and bipolar disorder, eating disorders, and dementia (Mellon & Griffin, 2002; Strous *et al.*, 2006). It is often difficult to determine whether the altered neurosteroid levels seen in some of these diseases is a *consequence* or *cause* of the condition, especially because some steroids have biphasic effects (e.g. pregnenolone sulphate is anxiolytic at low doses, but anxiogenic at higher doses (Strous *et al.*, 2006)). The roles for neurosteroids in anxiety and depression will be discussed in more detail below (*Sections 1.3 and 1.4*).

1.2.2. Neurosteroid binding to $GABA_A$ receptors

The effects of a range of neurosteroids and their analogues on $GABA_A$ receptor activity demonstrated stereospecificity and a biphasic action (potentiation at low concentration, activation at high concentration (Purdy *et al.*, 1990b; Paul & Purdy, 1992)). It was therefore proposed that there would be two specific neurosteroid binding sites on the $GABA_A$ receptor: an "activation site" and a "potentiation site". Identification of these sites was not a trivial task (see review by Hosie *et al.*, 2007), but was eventually achieved by electrophysiological characterisation of site-directed mutants expressed in HEK293 cells (Hosie *et al.*, 2006); the key residues for neurosteroid function are highlighted on *Fig. 1.4*. Note that with this updated $GABA_A$ receptor model, the activation site residues identified by Hosie *et al.* (2006), now point away from the interface between α and β subunits, where neurosteroid was proposed to bind. Indeed, more recent etomidate photolabelling suggests a model of the transmembrane domain (Li *et al.*, 2009; Olsen & Li, 2011) that is incompatible with that of Hosie *et al.* (2006). Residues βY284 and αT236 are therefore more likely to be involved in a transduction mechanism, rather than direct binding of neurosteroid. The orientations of residues in the potentiation site, on the other hand, are still compatible with the model of Hosie *et al.* (2006).

When heterologously-expressed receptors are studied, there are small isoform-dependent differences in responses that may be of relevance when considering the low nM concentrations of neurosteroids found *in vivo*: minimal potentiating doses for allopregnanolone were 3 nM at α1β1γ2 and α3β1γ2, 10 nM at α6β1γ2, and 30 nM at α2/4/5β1γ2 subunit combinations (Belelli *et al.*, 2002). Interestingly, receptors incorporating a δ subunit show increased efficacy relative to equivalent γ-containing combinations (Belelli *et al.*, 2002; Bianchi & Macdonald, 2003; Lambert *et al.*, 2003; Hosie *et al.*, 2009). However, this effect is probably not due to an interaction of neurosteroid with the δ subunit, because the potentiation site is entirely confined within the α subunit (Hosie *et al.*, 2009). Furthermore, a single point mutation of the conserved potentiation-site glutamine in the α4 subunit to leucine ($\alpha4^{Q246L}$) ablates neurosteroid potentiation

of α4β3δ receptors (Hosie *et al.*, 2009). The increased sensitivity of αβδ combinations is probably a reflection of the partial agonist nature of GABA at these receptors (c.f. αβγ receptors, where GABA is a full agonist) (Bianchi & Macdonald, 2003).

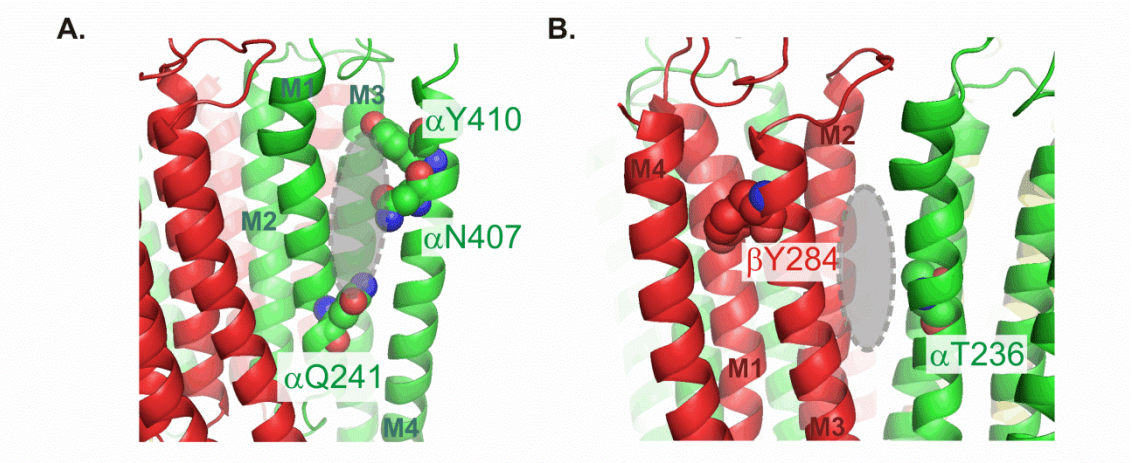

Figure 1.4 – $GABA_A$ receptor subunit assembly and neurosteroid binding sites

Ribbon diagrams depict the secondary structure of the transmembrane regions of α (green) and β (red) subunits, viewed in the plane of the membrane, and detail the proposed neurosteroid potentiation (A) and direct activation (B) sites. Structures are a 3D homology model of an α1β2γ2 $GABA_A$ receptor, based on the crystal structure of the *Caenorhabditis elegans* glutamate-gated chloride channel, GluCl (Hibbs & Gouaux, 2011), courtesy of Dr. Marc Gielen. Residues important for neurosteroid binding are highlighted as solid spheres, whilst the proposed neurosteroid binding pockets (Hosie *et al.*, 2006) are indicated by grey ovals. The potentiation site is contained within the α subunit, involving transmembrane helices M1 and M4 (key residues in α1 are Q241, N407 and Y410, which are conserved across all α subunit isoforms). Within this model, the position of key conserved residues in the direct activation site – α1 T236 and β2 Y284 – appear inconsistent with the proposed neurosteroid binding roles for these residues.

1.2.3. Physiological modulation of $GABA_A$ receptors by neurosteroids

The neurosteroids allopregnanolone and THDOC enhance GABAergic transmission by slowing the decay of IPSCs (Belelli & Herd, 2003; Harney *et al.*, 2003) and/or increasing the magnitude of tonic currents (Stell *et al.*, 2003). Some investigators have proposed that physiological (low nM) levels of neurosteroid will be ineffective at synaptic $GABA_A$ receptors – for example, Stell *et al.* (2003) found that DG GC tonic currents were sensitive to 10 nM THDOC, but phasic events only to 100 nM of this neurosteroid. Because tonic currents are often carried by αnβnδ combinations (e.g. in DG GCs (Nusser & Mody, 2002; Stell *et al.*, 2003) and cerebellar GCs (Brickley *et al.*, 2001)), they might be better poised to respond to endogenous steroids than synaptic αnβnγ combinations (Bianchi & Macdonald, 2003). However, there is some evidence for modulation of phasic events by physiological neurosteroid levels. Firstly, Puia *et al.* (2003) showed that decreasing endogenous allopregnanolone levels in neocortical brain slice tissue (using SKF-10511, an inhibitor of 5α-reductase type I and II) speeds the IPSC decay times recorded from pyramidal neurons. Furthermore, CA1 PC miniature IPSCs (mIPSCs) respond to 10 nM allopregnanolone (Harney *et al.*, 2003), whilst tonic currents in these cells appear neurosteroid-insensitive (Stell *et al.*, 2003). It therefore seems likely that, *in vivo*, endogenous neurosteroids will modulate both phasic and tonic currents, with the extent of modulation of each current depending on the neurosteroid-sensitivity of the underlying $GABA_A$ receptor composition.

Interestingly, the sensitivities of various neurons to neurosteroids do not always correlate with the relative sensitivities of the major $GABA_A$ receptor isoforms expressed in these cells (Belelli *et al.*, 2006). By a comparison of responses across the literature, it would seem that IPSC sensitivity to neurosteroid can vary with cell type (Harney *et al.*, 2003) and age of animal (Cooper *et al.*, 1999; Mtchedlishvili *et al.*, 2003), and may depend on differences in local neurosteroid metabolism (Belelli & Herd, 2003), receptor subunit composition (Brussaard *et al.*, 1997; Cooper *et al.*, 1999; Mtchedlishvili *et al.*, 2003) and relative kinase and phosphatase activities (Brussaard *et al.*, 2000; Fancsik *et al.*, 2000; Harney

et al., 2003; Koksma *et al.*, 2003). It is possible that some cells would respond to the basal *in vivo* levels of neurosteroids, whilst others will only be modulated by neurosteroids when concentrations are increased – by stress or pregnancy, for example (e.g. Harney *et al.* (2003) show that synaptic events in CA1 PCs respond to basal allopregnanolone levels (10 nM), whilst DG GCs will only respond to heightened levels (300 nM)).

As was discussed in *Section 1.1.3*, the effects of phosphorylation on $GABA_A$ receptor function appear to depend on the receptor subunit composition, the kinase, and the cell-type studied. These observations can be extended to kinase/phosphatase modulation of neurosteroid sensitivity. For example, Harney *et al.* (2003) attribute the differential allopregnanolone sensitivity (measured by IPSC decay prolongation) of hippocampal PCs and DG GCs to distinct activities of PKC and PKA within these cells. Constitutive activity of PKC and G protein appears to be required for allopregnanolone sensitivity in magnocellar neurons of the supraoptic nucleus (SON) of rats: IPSC decay prolongation by 1 µM of this neurosteroid is prevented by inhibitors of these proteins, whilst activators of these proteins do not further enhance the response (Fancsik *et al.*, 2000). Interestingly, the latter cell type is unaffected by inhibition of PKA, further supporting the notion that there is a cell-type variation in the kinases that affect neurosteroid modulation (i.e. PKA activity is more important in CA1 PCs (Harney *et al.*, 2003) than SON magnocellar neurons (Fancsik *et al.*, 2000)). Even within the same cell type, however, there is conflicting evidence for the effects of phosphorylation: unlike the work by Fancsik *et al.* (2000), others find that inhibition of PKC is required for neurosteroid sensitivity of SON magnocellar neurons and that constitutive activity of phosphatases PP1/PP2A determine allopregnanolone sensitivity of these cells during pregnancy (Brussaard *et al.*, 2000; Koksma *et al.*, 2003). Some of the discrepancies in the literature may relate to the use of broad-range kinase modulators (e.g. phorbol esters stimulate a range of PKC isoforms, which may have opposing effects on receptor function (Song & Messing, 2005)). GABAergic currents *in vivo* are probably modulated in concert by kinases/phosphatases and neurosteroids. For example, a combination of falling neurosteroid levels, and reduced neurosteroid sensitivity (by $GABA_A$ receptor

phosphorylation), are thought to contribute to the increased neuronal firing required for timed release of oxytocin at parturition (Brussaard *et al.*, 2000; Koksma *et al.*, 2003).

1.2.4. Neurosteroids: therapeutic potential

Historically, synthetic neurosteroid analogues were used for general anaesthesia during surgery (Prys-Roberts & Sear, 1980). Although the first evidence of anaesthetic action for neurosteroids came in the 1940s for progesterone (Paul & Purdy, 1992; Belelli *et al.*, 2006), it was not until the 1980s that this response was shown to involve an enhancement of $GABA_A$ receptor function (Harrison & Simmonds, 1984; Harrison *et al.*, 1987; Paul & Purdy, 1992). Despite their favourable potencies and safety profiles, these drugs are no longer used in humans due to the frequency of anaphylactic reactions, although these may be due to the Cremphor EL vehicle used (Prys-Roberts & Sear, 1980). There is resurging interest in translating neurosteroid-based therapies to the clinic because of their potency and array of favourable effects when studied in animal models: neurosteroids can be anxiolytic, antidepressant, analgesic, sedative, anticonvulsant, anaesthetic, and can suppress the action of the stress-responsive hypothalamic-pituitary-adrenal (HPA) axis (Carter *et al.*, 1997; Barbaccia *et al.*, 1998; Rodgers & Johnson, 1998; Khisti *et al.*, 2000; Winter *et al.*, 2003; Belelli *et al.*, 2006).

The clinical potential for neurosteroids has been well supported for treatment of Niemann Pick type C disease and epilepsy. The former is an inherited lysosomal storage disorder where patients suffer rapid neurodegeneration, which is ultimately fatal. Mouse models of Niemann Pick disease have very low allopregnanolone levels, and the administration of allopregnanolone delays the onset of motor dysfunction and extends survival (i.e. modulation of $GABA_A$ receptors by this neurosteroid is crucial for proper brain development and survival (Griffin *et al.*, 2004; Mellon *et al.*, 2008)). The anti-seizure activity of the

neurosteroid analogue, ganaxolone, has been extensively verified in animal models of epilepsy (Carter *et al.*, 1997; Reddy & Rogawski, 2009, 2010) and it has had some success in human clinical trials (Nohria & Giller, 2007). There is also anecdotal evidence favouring the use of ganaxolone in catamenial epilepsy (Reddy & Rogawski, 2009). In this subtype of epilepsy, sufferers experience an increase in seizure frequency and severity in response to an ovarian-cycle-linked fall in allopregnanolone levels, and an associated increase in expression of α4-type $GABA_A$ receptor subunits (Smith *et al.*, 1998; Reddy & Rogawski, 2009).

To establish and appreciate the full therapeutic potential of neurosteroids and their analogues, it is also important to fully define their normal physiological actions at molecular, cellular and systems levels. We predict that neurosteroid functions will be separable according to α subunit isoform (as was determined for benzodiazepines: see *Table 1.1*). Our intention was therefore to explore the physiological functions and therapeutic potential of neurosteroids in anxiety and depression. The following sections will outline the current understanding in these fields.

1.3. Neurosteroids and $GABA_A$ receptors in anxiety

1.3.1. The HPA axis and neurosteroids as endogenous anxiolytics

The HPA axis comprises a set of interactions between the hypothalamus, pituitary gland and adrenal cortex, and is activated by stress (Mody & Maguire, 2011). Activation of the axis stimulates production of a series of steroid hormones in the adrenal cortex, including allopregnanolone and THDOC. Activation of the rat HPA axis by exposure to stress, such as foot-shock or forced swim, or by inhibiting GABA synthesis or negatively modulating $GABA_A$

receptors, therefore induces an increase in brain and plasma neurosteroid levels (Purdy *et al.*, 1991; Barbaccia *et al.*, 1996; Barbaccia *et al.*, 1998; Vallee *et al.*, 2000). These rises in neurosteroid are disrupted by adrenalectomy and castration, indicating that increased production of these compounds occurs mostly in the periphery, rather than in the CNS (Purdy *et al.*, 1991; Barbaccia *et al.*, 1998). Indeed, after this surgery, basal and stress-associated levels of THDOC are undetectable (Purdy *et al.*, 1991). Basal allopregnanolone levels are also greatly lowered after adrenalectomy, but this steroid is still detectable in the cerebral cortex after such treatment, indicating another source for its production (probably the CNS) (Purdy *et al.*, 1991). By examining the time-courses of behavioural and neurochemical responses to acute stress (Purdy *et al.*, 1991; Barbaccia *et al.*, 1998), this HPA-induced rise in neurosteroids had been proposed to represent a feed-back mechanism to recover normal GABAergic tone and HPA function after acute stress. Given that injected neurosteroids reduce anxiety (Crawley *et al.*, 1986; Wieland *et al.*, 1991), the stress-induced production of neurosteroids is thought not only to limit the neurochemical response to stress, but also the behavioural response – i.e. neurosteroids probably function as endogenous anxiolytics (Barbaccia *et al.*, 1998).

The hippocampus is one of the main inputs to the hypothalamus that triggers HPA axis activation in stress, and is probably a key site for this neurosteroid-mediated feedback (by enhancing inhibitory neurotransmission in the hippocampus). Excessive activation of the HPA axis by chronic stress can be particularly damaging to the hippocampus, and reduces the number of parvalbumin-positive interneurons (Hu *et al.*, 2010). Diminished inhibitory neurotransmission within the hippocampus could therefore trigger pathological positive feedback – further activating the HPA axis (with glucocorticoids further damaging the hippocampus (Brown *et al.*, 1999; Hu *et al.*, 2010)). Such HPA axis dysregulation may account for the hippocampal atrophy observed in patients suffering from post-traumatic stress disorder and depression (Brown *et al.*, 1999; Sheline, 2003). Furthermore, the consequent impairment of regulation of network oscillations may underlie the cognitive defects in stress-related disorders (Hu *et al.*, 2010). We have therefore focussed part of our investigation

on inhibitory neurotransmission in the hippocampus, probing whether neurosteroid modulation of this transmission involves the α2-type $GABA_A$ receptor.

1.3.2. Defining the $GABA_A$ receptor α subunits that are important in anxiety and anxiolysis

An assortment of evidence is available to support a role for α2-type $GABA_A$ receptors in anxiety and anxiolysis. Firstly, this subunit is enriched in brain regions linked to emotion and anxiety, including the hippocampus and amygdala (Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002; Mohler, 2006a). Furthermore, α2 knock-out (α2-/-) mice are anxious in a conditioned emotional response paradigm (Dixon *et al.*, 2008) and $\alpha2^{H101R}$ knock-in mice (benzodiazepine-insensitive at α2-type $GABA_A$ receptors) lose the anxiolytic response to diazepam (Low *et al.*, 2000).

Not all investigators agree, however, that α2 subunits are the sole mediators of anxiolysis. In an investigation by Low *et al.* (2000), mice insensitive to benzodiazepine action at the α3 subunit retain anxiolysis following diazepam administration, and so investigators concluded that this subunit was not involved in anxiety. This notion is further supported by observations that α3-/- mice are not anxious and retain diazepam anxiolysis (Yee *et al.*, 2005). On the other hand, an α3-selective inverse agonist is anxiogenic (Atack *et al.*, 2005) and an α3-selective agonist is anxiolytic (Dias *et al.*, 2005), both of which implicate α3 subunits in anxiety circuitry. Notably, there are confounding issues, such as problems with activity effects in locomotor-dependent anxiety tests (see discussion by Reynolds *et al.*, 2001), or issues of imperfect selectivity of subunit-selective compounds (e.g. the α3-selective inverse agonist shows some efficacy at α2 subunits (Atack *et al.*, 2005), and so its anxiogenesis could be α2-mediated). Nevertheless, both α2 and α3 subunits mediate the myorelaxant effects of benzodiazepines: α2 subunits at low doses, and α3 subunits at higher

doses (Crestani *et al.*, 2001). It would seem probable that there is an overlapping contribution of both subunits in anxiolysis.

It is generally accepted that α1 subunits mediate the sedative effects of benzodiazepines (Rudolph *et al.*, 1999). Investigators therefore hoped that creating a α2/α3-selective benzodiazepine-like compound that has little activity at α1 subunits would be expected to produce anxiolysis without sedation. Several such compounds have been generated, including TPA023 (also called MK-0777) and MRK-409 (also called MK-0343), which have proven successful as non-sedating anxiolytics in pre-clinical assessments (Atack, 2008; Atack *et al.*, 2011). However, in spite of promising pre-clinical results, subunit-selective compounds have yet to be translated to the clinic. TPA023 failed at phase II clinical trials due to toxicity in long-term dosing (Mohler, 2011). MRK-409 unexpectedly proved sedative in humans, despite a lack of sedation in preclinical models; investigators believe this effect is a consequence of partial agonist efficacy at α1 subunits, but indicates that humans have a greater sedation tendency than animal models (Atack, 2010; Atack *et al.*, 2011). Confusingly, Ocinaplon is a compound that shows an anxioselective effect in humans and animal models, but has greater efficacy (both absolute and relative to diazepam) at α1- than α2-type $GABA_A$ receptors (Lippa *et al.*, 2005). Nevertheless, the compound TPA023 at least provides proof of principle that α2-selective/α1-sparing compounds can be non-sedating anxiolytics in human subjects (Atack, 2008).

We propose that neurosteroids will exhibit a similar α-subunit-selective profile, and thus predict a role for α2-type $GABA_A$ receptors in anxiolytic responses to both endogenous and injected neurosteroids. Furthermore, if this hypothesis is correct, α2-subunit-selective neurosteroid analogues would be non-sedating anxiolytics – opening an alternative drug design avenue to the selective benzodiazepine approach.

1.4. Neurosteroids and $GABA_A$ receptors in depression

1.4.1. Roles for $GABA_A$ receptors in depression

For many years, a prevailing theory for depression was the 'monoamine hypothesis', which postulates that depression results from a reduction in monoamine neurotransmission. Iproniazid and imipramine had demonstrated antidepressant efficacy in humans, and were later shown to increase serotonin and noradrenaline transmission – suggesting that deficiencies in these neurotransmitters underlie depression (see review by Krishnan & Nestler, 2008). Many antidepressant agents therefore aim to raise synaptic levels of monoamines, either by blocking degradation (e.g. tranylcypromine, which inhibits monoamine oxidases) or re-uptake into neurons (e.g. selective serotonin reuptake inhibitors (SSRIs)). Despite their widespread use, these drugs are not universally effective, and it is increasingly appreciated that the monoamine hypothesis has some shortcomings (Lacasse & Leo, 2005). Alternatives to the monoamine hypothesis are emerging, with additional modes of action being proposed for common antidepressants, as well as new suggestions for the aetiopathology of depression, including a role for GABAergic signalling.

Luscher *et al.* (2011b) and Smith and Rudolph (2012) reviewed the literature in support of a role for GABAergic dysfunction in depression. This connection might not be a surprise, given that anxiety and depression are often co-morbid (Hirschfeld, 2001; Nutt *et al.*, 2006), and that $GABA_A$ receptors are linked with anxiety (*see Section 1.3.2*). Co-morbid anxiety and depression is a significant challenge because patients often present with greater symptom severity and have delayed or diminished response to conventional treatment (Hirschfeld, 2001; Nutt *et al.*, 2006). A better understanding of the common mechanisms for anxiety and depression may therefore help develop better treatment strategies for such patients.

Arguably the best demonstration of a causative role for GABAergic deficits in anxiety and depression comes from study of heterozygous mouse knock-outs for the γ2 subunit of $GABA_A$ receptors (γ2+/-), which display phenotypes consistent with both human disorders, including increased avoidance of aversive environments, increased despair and enhanced sensitivity to ambiguous cues (Crestani *et al.*, 1999; Earnheart *et al.*, 2007). Furthermore, this mouse model recapitulates the HPA axis hyperactivity seen in melancholic forms of depression (Shen *et al.*, 2010). Particularly important for the purposes of this study, however, are observations that α2-/- mice have phenotypes consistent with both anxiety and depression (Dixon *et al.*, 2008; Vollenweider *et al.*, 2011) and that several limbic regions whose structure and/or activity is altered in depression, including the hippocampus, amygdala and basal ganglia (Sheline, 2003; McCabe *et al.*, 2009), are areas rich in $GABA_A$ receptor α2 subunit expression (Wisden *et al.*, 1992; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002; Mohler, 2006a).

There is also evidence from humans in support of a GABAergic signalling deficit in depression. A correlation between depressive symptoms and reduced brain levels of GABA was noted in cortical biopsies taken from fourteen depressed patients (Honig *et al.*, 1988). Although not necessarily representative of brain levels, plasma GABA concentrations can be more conveniently determined, and were also diminished in approximately 40% of depressed patients compared to healthy controls (Petty, 1994). Non-invasive means of measuring brain GABA levels were later developed, such as the proton magnetic resonance spectroscopic approach of Sanacora *et al.* (1999). Their work confirmed a GABA deficit in the occipital cortex (Sanacora *et al.*, 1999), and prefrontal regions (Hasler *et al.*, 2007) of depressed individuals, the latter of which is more likely to be of significance for mood disorders.

A role for $GABA_A$ receptors in human depression is supported by examining subunit mRNAs in post-mortem samples, mostly from depressed suicide victims. These studies found increases and decreases in expression of various

subunits, including α1, α3, α4 and α5 (Luscher *et al.*, 2011b). However, it is important to note that these mRNA fluctuations may not necessarily be paralleled by cell surface protein expression levels. By employing single photon emission computed tomography (SPECT) with [^{123}I]iomazenil to estimate the number of cortical $GABA_A$ receptors, Kugaya *et al.* (2003) found no change between 13 depressed patients and 19 healthy controls, which may negate the above findings with mRNA levels. Nevertheless, the SPECT approach is not without its limitations, particularly an inability to determine which subunit combinations make up the $GABA_A$ receptors to which [^{123}I]iomazenil is bound. Kugaya *et al.* (2003) therefore cannot rule out an upregulation of one subunit at the expense of another, despite no change in the overall number of [^{123}I]iomazenil binding sites. By using post-mortem slices of hippocampus from Bipolar 1 patients and healthy controls, Dean et al. (2005) were able to concur with such a mechanism: overall binding of [^{3}H]flumazenil was unaltered (i.e. no change in overall $GABA_A$ receptor expression), but the proportion of [^{3}H]flumazenil binding that was sensitive to displacement by zolpidem was lowered (note that, unlike [^{3}H]flumazenil, zolpidem does not bind to α5 subunits). The interpretation of these observations is therefore that Bipolar 1 patients have an increased expression of α5 subunits, at the expense of other α subunits in their hippocampi.

Several genetic association studies fail to link $GABA_A$ receptor α subunits with major depression and anxiety disorders (Henkel *et al.*, 2004; Pham *et al.*, 2009). However, these studies used a broad range of subjects with anxiety and depressive symptoms, and links may have been diluted by such generalisations; for example, Henkel *et al.* (2004) do find an association between $GABA_A$ α3 subunit and unipolar major depressive disorder specifically in female subjects. Furthermore, Yamada *et al.* (2003) find association between polymorphisms on genes encoding $GABA_A$ receptor α1 and α6 subunits and mood disorders in female patients. Horiuchi *et al.* (2004) also link an α1 subunit polymorphism with a cohort of Japanese patients suffering affective disorders.

Reductions in brain tissue volume in several areas in depression (reviewed by Sheline, 2003) could represent excitotoxic loss of neurons and/or a reduction in neurogenesis. Support for reduced neurogenesis comes from the γ2+/- mouse model of depression, where reduced γ2 expression impairs survival or differentiation of neuronal precursors into mature neurons (Earnheart *et al.*, 2007). Interestingly, the effects of reducing γ2 levels on hippocampal neurogenesis and animal behaviour depend on the developmental stage at which the deficit is imposed. A conditional knock-out of γ2 after the fourth postnatal week fails to recapitulate effects of losing this subunit during embryonic development (Earnheart *et al.*, 2007; Shen *et al.*, 2010). The story is not complete, however, since the developmentally-delayed loss of γ2 subunits in the model described above retains the HPA axis hyperactivity characteristic of depression (Shen *et al.*, 2010), but fails to develop the hippocampal neurogenesis defects and behavioural depression, that occurs if the γ2 deficit is imposed during embryogenesis (Earnheart *et al.*, 2007).

Given the above indications that depression involves reduced transmission through $GABA_A$ receptor subunits, one might predict that potentiating this transmission would be a successful treatment strategy. Indeed, Luscher *et al.* (2011b) suggest that current antidepressants, particularly SSRIs, may potentiate GABAergic transmission in a number of ways: by increasing excitability of GABAergic interneurons; by increasing GABA production via the enzyme, glutamate decarboxylase 67 (GAD67); and by increasing levels of $GABA_A$ receptor-potentiating neurosteroids (see *Section 1.4.2*). However, benzodiazepines – classical potentiators of GABAergic transmission – are not generally antidepressant; only alprazolam has demonstrated any efficacy in depression, although its use is mostly avoided due to its dependence liability (Laakmann *et al.*, 1996). More work clearly needs to be done to fully understand the roles $GABA_A$ receptor dysfunction in depression. We aim to contribute to this by further assessing the roles played by α2-type $GABA_A$ receptors in depression.

1.4.2. Roles for neurosteroids as antidepressants

Plasma and cerebrospinal fluid (CSF) levels of the neurosteroid allopregnanolone have repeatedly been shown to be reduced in depressed patients relative to healthy controls (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000). Furthermore, this allopregnanolone deficit is ameliorated by treatment with a range of antidepressants, with the recovery of CSF levels (Uzunova *et al.*, 1998), but not plasma levels (Romeo *et al.*, 1998), correlating with the degree of symptomatic resolution. By characterising the effects of SSRIs on rat brain neurosteroid profiles (Uzunov *et al.*, 1996) and on the kinetics of recombinantly-expressed enzymes (Griffin & Mellon, 1999), the increased allopregnanolone concentration appears to result from stimulation of the enzyme 3α-HSD (see *Fig. 1.3*) changing its properties to favour the reductive reaction over the oxidative reaction. It would therefore seem that neurosteroids could be used as antidepressants. Importantly, however, such correlative observations do not demonstrate whether neurosteroid level normalisation is a *consequence* or *cause* of diminished depressive symptoms. Support for a causative role is provided by observations that sex hormones and their neurosteroid metabolites modulate behaviour in several rodent models of depression, including forced swim, tail suspension and learned helplessness tests (Khisti *et al.*, 2000; Hirani *et al.*, 2002; Dhir & Kulkarni, 2008; Shirayama *et al.*, 2011). Antagonism of allopregnanolone's antidepressant effect by bicuculline implicates $GABA_A$ receptors as the mediators of the response to neurosteroids (Khisti *et al.*, 2000).

At childbirth, the sudden withdrawal from pregnancy-associated high levels of progesterone, and its neurosteroid metabolites, is thought to trigger post-partum depression in susceptible women (Bloch *et al.*, 2000). Support for these proposals comes from progesterone-withdrawal mouse models of post-partum depression, where repeated progesterone injections are followed by either a progesterone receptor antagonist or finasteride (an inhibitor of 5α-reductase) to inhibit further metabolism to neurosteroids. The former demonstrates that some of the depressed phenotype involves the progesterone receptor (Beckley *et al.*,

2011), but the latter confirms that reduced levels of its metabolites, especially allopregnanolone, also contribute to the depressed phenotype in the forced swim test (Beckley & Finn, 2007). Mouse models also support a role of $GABA_A$ receptors in postpartum depression: δ+/- and δ-/- mice are depressed in various behavioural tests, but only post-partum – virgin females show no phenotype – which suggests a protective role of δ subunit expression (Maguire & Mody, 2008).

Whilst $GABA_A$ receptor potentiation by neurosteroids may account for some antidepressant-like effects, there are some important points to note. SSRI antidepressants are slow to act in humans, requiring several weeks of dosing before a beneficial effect is observed, whilst their effects in rodent behavioural models of depression are much more immediate, occurring within minutes (Cryan *et al.*, 2005). In addition, although these drugs rapidly alter 3α-HSD activity and brain neurosteroid profiles in rats (Uzunov *et al.*, 1996; Griffin & Mellon, 1999), chronic fluoxetine treatment in rats actually decreases the plasma and brain concentrations of THDOC and allopregnanolone (Serra *et al.*, 2002). Furthermore, other antidepressants, such as imipramine, do not influence 3α-HSD activity or neurosteroid levels in rodents (Uzunov *et al.*, 1996; Griffin & Mellon, 1999). Finally, sulphated steroids, which negatively modulate $GABA_A$ receptors, are also antidepressant in mice (Dhir & Kulkarni, 2008). Therefore, although neurosteroid potentiation at $GABA_A$ receptors can produce antidepressant-like effects in animal models, they are by no means a universal feature of antidepressant function. We therefore aim to screen the antidepressant potency of the neurosteroid THDOC, and assess whether this function depends on positive allosteric modulation of α2-type $GABA_A$ receptors.

1.4.3. The nucleus accumbens in reward and depression

The nucleus accumbens (NAcc) forms part of the mesolimbic dopamine pathway, which is believed to process rewarding stimuli, and to be involved in

motivational reward-seeking pathways. Major inputs to this structure are dopaminergic projections from the ventral tegmental area (VTA), glutamatergic projections from the hippocampus, as well as inputs from prefrontal association cortices and the basolateral amygdala (Heimer *et al.*, 1997). Drugs of abuse, including cocaine and benzodiazepines, engender rewarding effects by increasing dopamine release in the NAcc, often by relieving inhibitory restriction on VTA dopamine neuron firing (Hyman & Malenka, 2001; Luscher & Ungless, 2006; Tan *et al.*, 2011). NAcc activity is under extensive inhibitory control: the vast majority (95%) of neurons in the NAcc are GABAergic medium spiny neurons (MSNs), which not only project to outputs (such as the ventral pallidum), but also make collateral connections with one another (Heimer *et al.*, 1997). Changes in GABAergic transmission may occur during addiction and withdrawal – for example, the level of extracellular GABA increases in the rat NAcc after withdrawal from daily cocaine injections (Xi *et al.*, 2003).

The mesolimbic dopamine pathway is not only involved in response to addictive drugs, but is also activated by a number of natural rewards, such as food. Anhedonia, a reduced pleasure in response to normally rewarding stimuli, is a core symptom of depression, suggesting that a defective mesolimbic dopamine pathway can underlie some features of depression (Shirayama & Chaki, 2006). It is possible that the antidepressant function of neurosteroids could involve their activity within the mesolimbic dopamine system. Direct injection of allopregnanolone into the NAcc was not antidepressant in a learned helplessness model of depression (Shirayama *et al.*, 2011), but these experiments have not definitively ruled out a role for the NAcc in antidepressant functionality of endogenously-synthesised neurosteroids. We have therefore examined inhibitory neurotransmission within the NAcc, and its response to neurosteroid. The roles of the $GABA_A$ receptor α2 subunit in this response were also assessed.

1.5. Thesis Aims

1.5.1. Generation of an α2^{Q241M} knock-in mouse

Hydrophobic substitution of a critical α1^{Q241} residue (or equivalent in other α subtypes) selectively disrupts neurosteroid binding to the potentiation site, eliminating neurosteroid potentiation, with minimal effects on GABA activation and receptor modulation by other allosteric ligands (barbiturates, benzodiazepines) (Hosie *et al.*, 2006; Hosie *et al.*, 2009). We have therefore used such a mutation to generate a transgenic knock-in mouse line that harbours the mutation α2^{Q241M}. By ablating neurosteroid potentiation at α2-type $GABA_A$ receptors, this knock-in mutation allows us to examine the α2-subunit specific functions of neurosteroids.

Especially because this mutation is influencing the response to an endogenous compound, one must verify a lack of compensatory alterations in the knock-ins. We have addressed this question using Western blot and quantitative immunofluorescence assays to study $GABA_A$ receptor α subunit expression. Because this mutation is not expected to alter diazepam or pentobarbital sensitivity of mutant channels compared to wild-types, we can utilise these compounds as positive controls within this study, to demonstrate that the effects of $GABA_A$ receptor potentiation are intact within the transgenic animals.

1.5.2. Electrophysiological characterisation of α2^{Q241M} mice

The roles of the α2 receptor isoform in the cellular responses to neurosteroids have been examined with whole cell patch-clamp recordings in acute brain slice tissue. The α2 subunit has been classically associated with synaptic events, IPSCs (Prenosil *et al.*, 2006), but we cannot rule out a role in extrasynaptic tonic currents, and so both types of inhibition have been assessed. The areas chosen

for study, the hippocampus and NAcc, strongly express the α2 isoform, and are key candidates for roles in anxiety and depression. To screen for effects of the mutation on baseline neurotransmission, periods of control recording were compared between wild-type and $\alpha2^{Q241M}$ knock-in mice. Slices were also treated with diazepam or THDOC, to evaluate the effect of the knock-in mutation on sensitivity to these compounds.

1.5.3. Behavioural characterisation of $\alpha2^{Q241M}$ mice

In this study, analyses have focussed on anxiety and depression-related phenotypes, to assess the contribution of neurosteroids and α2-type $GABA_A$ receptors to these disorders. We propose that neurosteroids may exert their anxiolytic and antidepressant actions through α2 and α3-type $GABA_A$ receptors. If our hypotheses are correct, homozygous $\alpha2^{Q241M}$ mutant mice would be predicted to show impaired anxiolysis and antidepression in response to injected neurosteroid. Behavioural phenotypes of untreated mice would also provide insight into the functions of endogenous neurosteroids acting at α2-type $GABA_A$ receptors.

1.5.4. Summary of thesis aims

1. To establish that the $\alpha2^{Q241M}$ mutation has the desired properties for this study: loss of neurosteroid potentiation without altered GABA sensitivity or benzodiazepine-mediated potentiation (Chapter 3).

2. To establish that $\alpha2^{Q241M}$ knock-in mice have no compensatory changes in receptor expression (Chapter 3).

3. To examine the consequences of $\alpha2^{Q241M}$ knock-in for inhibitory neurotransmission and its response to modulators (Chapter 4).

4. To define the $GABA_A$-receptor α2-specific roles of endogenous and injected neurosteroids in anxiety and depression-type behaviours (Chapter 5).

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Reagents

EDTA-Free Halt Protease Inhibitor Cocktail (100x) was purchased from Thermo Fisher Scientific Inc. (Rockford, Illinois, USA); as were the bicinchoninic acid (BCA) Assay Kit and the SuperSignal West Pico Chemiluminescent Substrate. Protogel 30% w/v Acrylamide:0.8% w/v Bis-Acrylamide was purchased from National Diagnostics (Atlanta, Georgia, USA). Protein Molecular Weight Standards (Broad Range) were purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, UK). Polymerase Chain Reaction (PCR) was performed using the Phusion Hot Start High-Fidelity DNA Polymerase kit (Thermo Fischer Scientific Inc.) and primers from Eurofins MWG Operon (Ebersberg, Germany). All other chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) or VWR International (Leuven, Belgium) unless indicated otherwise.

2.1.2. Antibodies

Table 2.1 provides details regarding sources and working dilutions for all primary and secondary antibodies used in this study. The specificity of anti-$GABA_A$-receptor antibodies used in Western blotting was confirmed using recombinantly expressed receptors. Briefly, Human Embryonic Kidney 293 (HEK293) cells were electroporated as described in *Section 2.5.2*. Two days later, cells were washed in ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and scraped off the culture dish in ice-cold RIPA buffer (150 mM NaCl, 20 mM Tris pH 8.0, 5 mM ethylene-diamine-tetra-acetic acid (EDTA), 1% v/v NP40, 0.5% w/v sodium

deoxycholate, 0.1% w/v sodium dodecyl-sulphate (SDS), 1 mM phenylmethane-sulfonylfluoride and 1x EDTA-Free Halt Protease Inhibitor Cocktail). Cells were disrupted by rotating in the RIPA buffer (1 h at 4 ^{o}C); debris was removed by sedimentation (30 min, 13,000 rotations per minute (r.p.m.), 4 ^{o}C, Eppendorf 5415R centrifuge). Further processing (BCA assay and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)) was performed as described in *Section 2.3.2*. Each antibody tested produced a band between markers 45 and 66 kiloDaltons (kDa), in the appropriate lane only (*Fig. 2.2*). Using the defined blotting conditions (*Section 2.3.2*) and antibody dilutions (*Table 2.1*), it was therefore evident that that these antibodies are $GABA_A$ α-subunit isoform specific.

2.2. Animals

All procedures involving animals were performed according to the *Animals (Scientific Procedures) Act, 1986* (ASPA) and had obtained local ethical approval. When obtaining tissue for electrophysiology or Western blotting, animals were decapitated under terminal isoflurane anaesthesia. After behavioural testing, animals were culled by cervical dislocation according to Schedule 1 of the ASPA. Given that the steroid hormones of the oestrus cycle have a strong influence on brain neurosteroid levels in female mice (Corpechot *et al.*, 1997), only male mice were used in our experiments.

	Antibody	WB dilution	IF dilution	Epitope Details	Source
Primary Antibodies	mouse monoclonal anti-$GABA_A$ α1	1:1,000	-	Amino acids 355-394 of mouse $GABA_A$ α1	NeuroMab [1]
	rabbit polyclonal anti-$GABA_A$ α1	-	1:20,000	Amino acids 1-16 of rat $GABA_A$ α1	gift from Dr Jean-Marc Fritschy [2]
	guinea-pig polyclonal anti-$GABA_A$ α2	-	1:1,000	Amino acids 1-9 of rat $GABA_A$ α2	gift from Dr Jean-Marc Fritschy [2]
	guinea-pig polyclonal anti-$GABA_A$ α2	1:1,000	-	Amino acids 29-37 of rat $GABA_A$ α2	Synaptic Systems [3]
	rabbit polyclonal anti-$GABA_A$ α2	1:500	-	Amino acids 1-10 of rat $GABA_A$ α2	gift from Dr Werner Sieghart [5]
	rabbit polyclonal anti-GABAA α3	1:1,000	1:1,000	Amino acids 29-37 of human $GABA_A$ α3	Alomone Labs [4]
	rabbit polyclonal anti-$GABA_A$ α4	1:4,000	1:500	Amino acids 1-14 of rat $GABA_A$ α4	gift from Dr Werner Sieghart [5]
	rabbit polyclonal anti-$GABA_A$ α5	-	1:500	Amino acids 1-12 of rat $GABA_A$ α5	gift from Dr Werner Sieghart [5]
	mouse monoclonal anti-β-Tubulin	1:1,000	-	Tubulin from rat brain	SigmaAldrich [6] (clone TUB2.1)
Secondary Antibodies	Alexa Fluor® 555 goat anti-guinea pig IgG (H+L)	-	1:2,000	-	Invitrogen Ltd. [7]
	Alexa Fluor® 555 goat anti-rabbit IgG (H+L)	-	1:2,000	-	Invitrogen Ltd. [7]
	HRP-conjugated goat anti-rabbit IgG (H&L)	1:10,000	-	-	Rockland Immunochemicals for Research [8]
	HRP-conjugated goat anti-mouse IgG(H&L)	1:10,000	-	-	Rockland Immunochemicals for Research [8]
	HRP-conjugated donkey anti-guinea-pig IgG(H&L)	1:1,000	-	-	Jackson ImmunoResearch Laboratories [9]

Table 2.1 – Details of antisera employed in this project

Details of antisera utilised in this project for Western blot (WB) or immunofluorescence (IF). Secondary antibodies for WB were conjugated to Horseradish peroxidise (HRP).[1] UC Davis, University of California, USA; [2] University of Zurich, Switzerland; [3] Goettingen, Germany; [4] Jerusalem, Israel; [5] Medical University Vienna, Austria; [6] Steinheim, Germany; [7] Paisley, UK; [8] Philadelphia, Pennsylvania, USA; [9] Stratech Scientific, Suffolk, England

2.2.1. Generating $GABA_A$ receptor $\alpha2^{Q241M}$ mutant mice

Mike Lumb, a molecular biology technician in the lab, performed this stage of the project. *Escherichia coli*-based recombineering technology (obtained from National Cancer Institute at Frederick, Maryland) was employed to create a targeting vector containing exon 8 of $GABA_A$ receptor α2 subunit gene with base pair changes for the point mutation Q241M (*Fig. 2.1 Ai*), utilising an RP23 Bacterial Artificial Chromosome library clone derived from a C57BL/6 mouse. Using commercial facilities for homologous recombination (GenOway, Lyon, France), the vector was introduced into 129Sv/Pas embryonic stem (ES) cells by electroporation. Positive and negative selection procedures were used to enrich for cells that had successfully undergone homologous recombination. PCR and Southern blotting techniques were used to identify ES cell lines that had precisely replaced the wild-type α2 sequence with that encoding the Q241M mutant: the mutagenesis silently created an Nco-I restriction site, allowing identification of homologous recombinants by Nco-I digestion followed by Southern blot (band size decreases from 12 Kilobase-pairs (kb) to 6 kb (*Fig. 2.1 B*)). These ES cell lines were used to generate transgenic mice with the $\alpha2^{Q241M}$ mutation (*Fig. 2.1 Aii*). Germline-transmitted pups from a chimera x C57BL/6J cross (F0 generation) were bred with C57BL/6J Cre-recombinase expressing mice, in order to remove the neomycin resistance cassette (F1 generation; *Fig. 2.1 Aiii*). Further backcrosses were performed between heterozygous (het) $\alpha2^{Q241M}$ mice and C57BL/6J mice. The mice used in the experiments described here are from generations F4-F6.

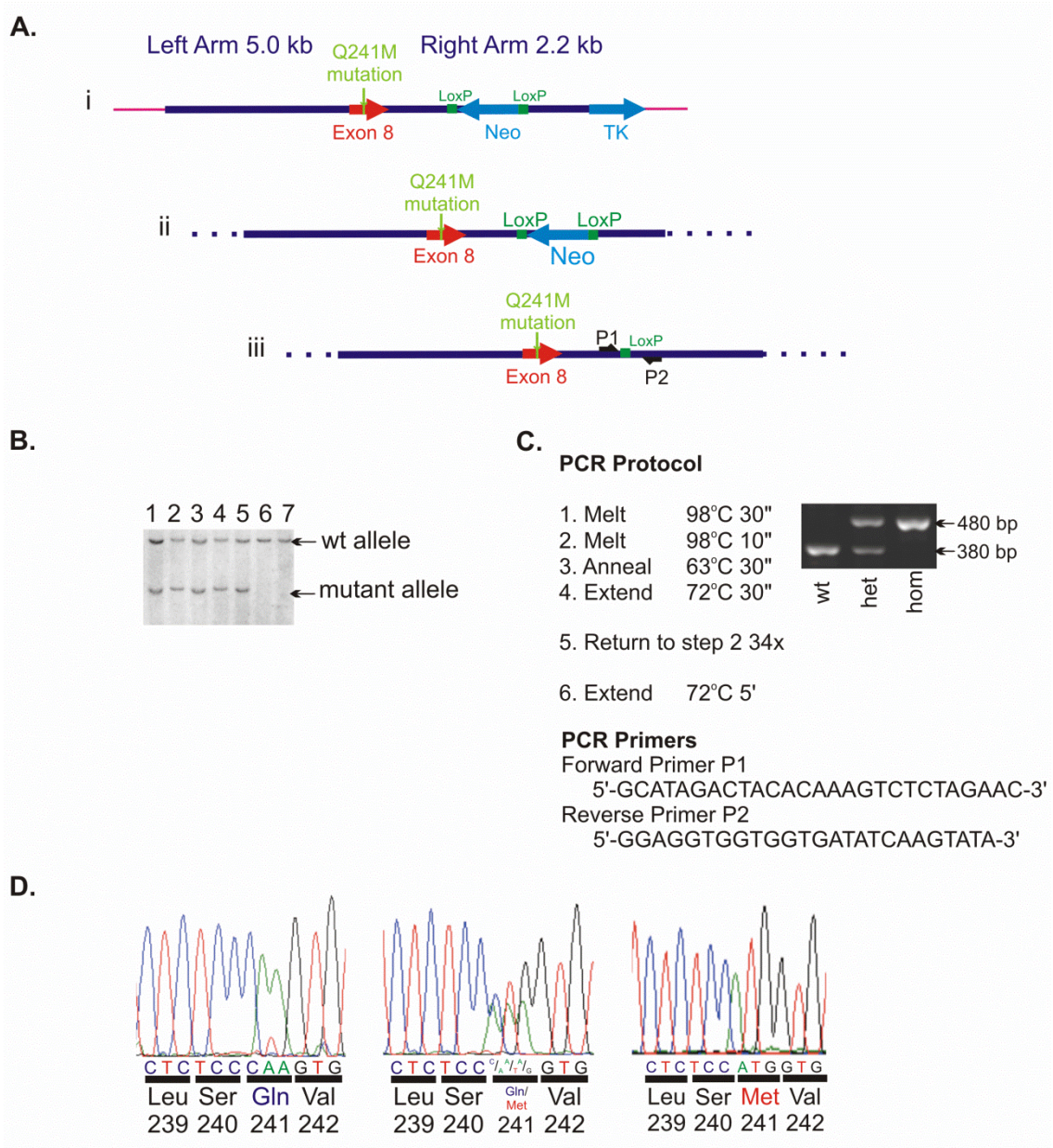

Figure 2.1 – Generation of the mutant mice

A. i. Targeting construct comprising mouse genomic sequence (dark blue) containing point-mutated exon 8 of $GABA_A$ α2 gene (red), together with positive and negative selection markers Neo (neomycin resistance cassette, light blue) and TK (*Herpes simplex* virus thymidine kinase, light blue). The Neo cassette is flanked by loxP sites (green). This construct is housed in a low copy number pBluescript KS+ plasmid backbone (pink).

ii. Result of successful homologous recombination; mutated exon 8 and neo have been incorporated into genomic DNA (dashed dark blue line). TK and pBluescript are lost. This corresponds to the genomic DNA from F0 generation mice.

iii. Result of breeding recombinant mice with Cre-recombinase expressing mice; Neo has been removed, leaving behind a single lox P site. Black arrowheads mark locations of primer sites used for genotyping in C. This corresponds to genomic DNA in mice from generation F1 and beyond.

B. Southern blot screen of ES cell lines: the Q241M mutation silently introduces a novel restriction site – allowing a screen for restriction fragment length polymorphism. Lanes 1-5, heterozygous recombinants; lanes 6-7, wild-type controls.

C. Primers and protocol for PCR genotyping of mouse genomic DNA, with representative results achieved by running PCR products on a 2% w/v agarose gel.

D. Verification of the point mutation by DNA sequencing. Exon 8 was PCR-amplified from mouse genomic DNA of wt (left), het (middle) and hom (right) animals, then sequenced. Sequence results local to the point mutation are shown.

2.2.2. Breeding

Animals were maintained as het x het breeding pairs, to allow comparison of homozygous (hom) knock-ins with wild-type (wt) littermates. Experimental animals were males housed in cages of up to five littermates, with access to food and water *ad libitum*, under a 12 h light-dark regime (lights on at 07:30h). Males were housed with at least one other littermate post-weaning. For electrophysiology and Western blotting, animals were used between postnatal days 18 (P18) and 30. For behavioural characterisation and immunofluorescence, animals aged between P42 and P70 were used.

2.2.3. Genotyping

Genomic DNA was isolated from ear clips and/or post-mortem tail snips by incubating overnight in a lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% w/v SDS, 200 mM NaCl and 0.1 µg/ml proteinase K (Roche Diagnostics GmBH, Mannheim, Germany)) at 37 ^{o}C. Debris was removed by centrifugation

(15 min, 13,000 r.p.m., room temperature (RT)); DNA was precipitated with isopropanol (sedimented at 13,000 r.p.m., 5 min, RT), washed with 70% v/v ice-cold ethanol (sedimented at 13,000 r.p.m., 5 min, RT), and re-suspended in 0.05x TE (10 mM Tris, 1 mM EDTA pH 8.0).

The mutant allele can be distinguished from the wt allele by the presence of the loxP site remaining after removal of the neomycin cassette on the transgenic allele (*Fig. 2.1 Aiii*). When PCR is performed as outlined in *Fig. 2.1 C*, the presence of the lox P site results in a band 100 base pairs larger in the mutant allele than the wt allele. A heterozygous mouse therefore produces two bands (*Fig. 2.1 C*).

2.3. Western blotting

2.3.1. Protein isolation

Total protein was isolated from four brain areas from each animal: cortex, cerebellum, hippocampus and nucleus accumbens (NAcc). The NAcc was dissected from 350 µm coronal slices obtained as for electrophysiology (for details, see *Section 2.6.1*). Remaining brain areas were isolated by direct dissection from whole brain under a dissecting microscope, and the meninges removed. Tissue was disrupted by homogenisation in ice-cold RIPA buffer, either in a Dounce homogeniser (VWR International) (cortex, hippocampus, cerebellum) or with a Microlance 25-gauge 1/2” needle and 1 ml syringe (BD Franklin Lakes, NJ, USA) (NAcc). Cells were disrupted by repeated freeze-thaw cycles, with debris removed after each cycle by centrifugation (20 min, 13000 r.p.m., 4 ^{o}C).

2.3.2. Polyacrylamide gel electrophoresis (PAGE) and blotting

Protein concentration was determined spectrophotometrically (Bio-Rad SmartSpec Plus) according to instructions with the BCA assay kit. Proteins were denatured at RT by addition of Laemmli Sample Buffer (150 mM Tris-Cl pH 6.8, 6% w/v SDS, 0.3% w/v Bromophenol blue, 30% w/v glycerol and 15% v/v β-mercaptoethanol) and run on a 10% SDS-PAGE gel (5% stacking gel) in Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for 1 h at 150 V, using a Bio-Rad Miniprotean II system. Proteins were transferred to nitrocellulose membranes (Hybond C Extra, Amersham Biosciences, Buckinghamshire, UK) at 25 V for 70 min in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) using an XCell II Blot Module (Invitrogen, Carlsbad, USA). Successful transfer was confirmed by Ponceau staining (0.1% w/v Ponceau-S in 5% v/v acetic acid); the stain was thoroughly washed off the membrane with PBS before the blocking and incubation steps.

All blocking, washing and antibody incubation steps were performed on a shaker with 4% milk in PBS (plus 0.1% TWEEN-20), the antibody dilutions employed are outlined in *Table 2.1*. Blocking was performed for 1 h at RT, whilst primary antibody incubation was overnight at 4 ^{o}C, and secondary antibody incubation was for 2 h at RT. After each antibody incubation, membranes were washed three times (20 min, RT). Blots were finally rinsed in PBS and developed using chemiluminescent substrate and visualised with an ImageQuant LAS4000 imager. Images were quantified using the Western blot plug-in on ImageJ software (Version 1.44p, National Institutes of Health, USA).

After blotting for the relevant $GABA_A$ α subunit, blots were subjected to a mild stripping procedure (10-20 min incubation in a buffer comprising 200 mM glycine, 0.1% w/v SDS, 1% v/v TWEEN-20, pH 2.2) followed by 6 x 5min washes in PBS. Successful stripping was confirmed by re-incubation in secondary antibody (1 h at RT), washing (3 x 20 min, RT) and developing, where no residual signal was observed. Membranes were then re-blotted for quantification of β-tubulin expression. The density of each α subunit band was

first normalised to its corresponding β-tubulin band, then signals within each Western blot were normalised to the average signal of the wt bands in that blot (such a normalization procedure has been used by others – e.g. Chandra *et al.*, 2005).

Before quantitative blots were carried out, titration experiments were performed to determine the amount of total protein, which, when blotted under the above conditions, produces a signal in the middle of the dynamic range of the detection system. When working under such conditions, one maximises the chances of detecting any up- or down-regulation of $GABA_A$ subunit expression. Gels were therefore loaded with increasing amounts of total protein (ranging from 5 to 100 µg) from each brain area from three P21-P30 C57BL/6J mice and blotted for each $GABA_A$ α subunit. These titration curves (*Fig. 2.2* and data not shown) were used to select an appropriate amount of protein to load when quantifying subunit expression from the transgenics (*Table 2.2*).

Subunit	Cortex	Hippocampus	Nucleus Accumbens	Cerebellum
α1	15 µg	25 µg	25 µg	20 µg
α2	25 µg (gp)	25 µg (gp)	20 µg (r)	n.d.
α3	45 µg	25 µg	20 µg	n.d.
α4	30 µg	n.d.	n.d.	n.d.

Table 2.2 – Amount of total protein loaded for Western blotting

Results from titration experiments determining the amount of total protein, which, when blotted under the defined conditions, produces a signal in the middle of the dynamic range of the detection system. Two different antisera were used in detecting α2 expression – guinea pig (gp) or rabbit (r) as indicated. n.d. - not determined because no reliable signal was obtained with calibration blots (0-100 µg total protein loaded).

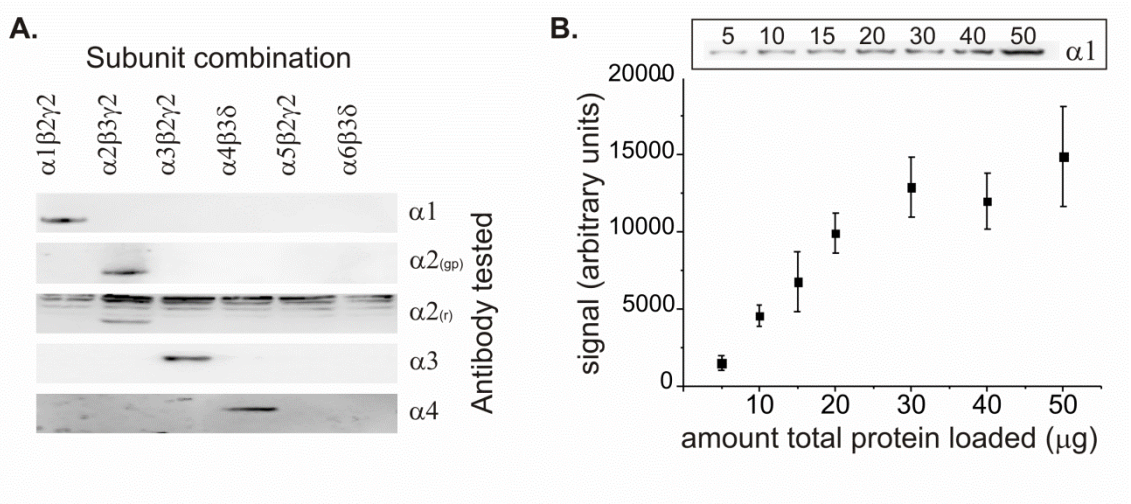

Figure 2.2 – Optimising conditions for Western Blotting

A. Antibodies are specific for their defined α subunit: 20 µg of each HEK293 cell lysate was prepared and blotted as described in *Section 2.1.2*. Images show the blot between 66 kilodalton (kDa) and 45 kDa markers. Antibodies: α1, mouse monoclonal anti-α1; α2(gp), guinea-pig polyclonal anti-α2; α2(r), rabbit polyclonal anti-α2, α3, rabbit polyclonal anti-α3; α4, rabbit polyclonal anti-α4.

B. Calibration curve for the signal (arbitrary units) obtained by blotting increasing amounts of C57BL/6J cortical protein for α1; average of 3 blots (error bars = standard error of the mean (s.e.m.)). Loading 15 µg total protein from test cortices will give a mid-range signal. *Inset:* representative example blot (bands 5-50 µg correspond to the points on the curve below). Similar calibration curves were obtained for other subunits and other brain areas.

2.4. Immunofluorescence

2.4.1 Brain sectioning for immunofluorescence

Three mice of each genotype (wt, het, hom) were sacrificed with a lethal dose of sodium pentobarbital (dissolved in 0.9% w/v NaCl to a concentration of 15 mg/ml, injected at a volume of 10 ml per kg body weight), and subjected to cardiac perfusion first with 20 ml ice-cold saline/heparin mix (5000 units heparin

per litre (Leo Laboratories Ltd., Buckinghamshire, UK), 0.9% w/v NaCl), then with 10 ml ice-cold fixative (4% paraformaldehyde in 0.1 M Phosphate Buffer (PB: 12 mM NaH_2PO_4, 38 mM Na_2HPO_4)). Brains were removed, and incubated for a further 2 h at 4 ^oC in the fixative. Finally, they were transferred to a cryoprotectant solution comprising 0.1 M PB, 0.03% w/v sodium azide and 30% w/v sucrose for incubation at 4^oC until tissue sank (at least overnight).

Coronal 40 µm slices were cut from frozen brain on a Leica SM200R sliding microtome (Leica Microsytems GmBH, Wetzlar, Germany). Sections were collected in a 24-well plate (Starstedt Ltd., Leicester, UK), filled with 0.1M PB with 0.03% w/v sodium azide, and were stored at 4 ^oC until staining. During sectioning, serial sections were inserted into adjacent wells such that one well contains every 6th slice of the nucleus accumbens, or every 12th slice of the hippocampus. When staining, sections were taken from the same well, thus ensuring a uniform representation along the anterior-posterior axis of the structure being examined.

2.4.2. Staining sectioned tissue

Tissue sections were rinsed twice with PBS before incubating for 1 h at RT in permeabilisation/blocking solution (0.5% bovine serum albumin, 2% normal goat serum, 0.2% triton-x-100 in PBS). Sections were incubated overnight at 4 ^oC with primary antibody dissolved in the same permeabilisation/blocking solution. Secondary antibody was applied to slices at RT for 2 h. After each antibody incubation, slices were washed four times in PBS (15 min at RT). All incubations and washes were performed by gently shaking slices in 24 well-plates. Stained slices were mounted on super premium glass microscope slides (VWR International) using ProLong Gold Antifade reagent (Invitrogen Ltd. (Paisley, UK)). Slides were stored in a dark container at 4 ^oC, until imaging. Controls for non-specific labelling by secondary antibodies were performed by staining as described, but omitting primary antibody.

2.4.3. Image acquisition and analysis

Image acquisition was performed using a Zeiss Axioscop LSM510 confocal microscope (Carl Zeiss Ltd., Welwyn Garden City, Hertfordshire, UK), equipped with three laser lines (λ = 488, 543 and 643 nm) and Plan Neofluor 20x air (numerical aperture (NA) 0.5) and 63x oil (NA 1.4) differential interference contrast objectives (Carl Zeiss). Images were captured as z-stacks, with images in each plane acquired as a mean of 8 scans in 8 bits and stored for analysis. For each subunit and brain area, three slices were imaged per animal. Image acquisition and analysis were performed blind to the genotype of the animal.

Images of the *cornu ammonis 1* (CA1) region of the hippocampus were acquired with the 63x lens at 1x zoom, and encompass the cell body layer and the apical dendrites. Images of the dentate gyrus (DG) were also acquired with the 63x lens at 1x zoom, and encompass the granule cell layer and molecular layer of the medial blade of the DG. With the exception of images for α3 expression, images for the NAcc were acquired with the 63x lens at 1x zoom, and represent both core and shell regions. Because the expression of α3 was much less uniform throughout the NAcc, showing patches of intense staining in both core and shell regions, images for this subunit were instead acquired using the 20x lens at 1x zoom. This reduces the spatial resolution of the images, but acquires data over a 9.9 times larger surface area, and so compensates for this 'patchy' distribution.

Images were analysed using ImageJ (Version 1.44p). For CA1 and DG, the mean fluorescence intensity was determined for separate regions of interest (ROIs) encompassing the dendrites and cell bodies. For the NAcc, mean fluorescence intensity was measured for the entire image, rather than a specific ROI. In both cases, to ensure that the value is representative of the staining throughout the brain slice, three readings were taken per image: one from the

top, one central and one at the bottom of the z-stack; the average of these values was taken to represent the expression of that subunit in that slice.

Bar charts in *Fig. 3.5* represent the average mean fluorescence intensity across the whole collection of slices. Results are expressed relative to values for wt animals, such that het or hom values of 1.0 would represent no change in expression; and, for example 1.2 would represent a 20% increase, or 0.8 a 20% decrease in expression.

The mean fluorescence intensity approach is less appropriate in images where punctae are in the minority against background (e.g. see α1 expression in the NAcc, *Fig. 3.4 C*). In this case, and also for α3 expression in the DG (where the mean fluorescence intensity analysis indicated a tendency toward a difference), subunit expression was examined in more detail by quantifying immunopositive punctae. This was carried out using the FociPicker3D plug-in in ImageJ (Guanghua Du, Institute of Modern Physics, CAS, China), which searches for local maxima in three-dimensions. As demonstrated in *Fig. 2.3 A*, when using optimal settings (intensity and size thresholds to define positive foci against the local background), the program successfully picks out immunopositive punctae, and provides measurements of their volume and intensity.

The FociPicker3D approach was not extended to other images for several reasons. The plug-in is not suitable for images containing a mixture of diffuse and punctate staining (e.g. α4 and α5 subunit immunofluorescence), because it fails to distinguish between a small punctae and diffuse immunofluorescence (see *Fig. 2.3 B*). The bright but diffuse staining of some dendritic processes in hippocampi stained with α1-selective antiserum poses a similar problem (being recognised as a large number of small punctae by FociPicker3D). In addition, no settings were found that enabled the FociPicker3D plug-in to satisfactorily identify α2 immunopositive punctae in any of the regions imaged, probably because of the high background fluorescence in these images (which may represent diffuse staining for this subunit).

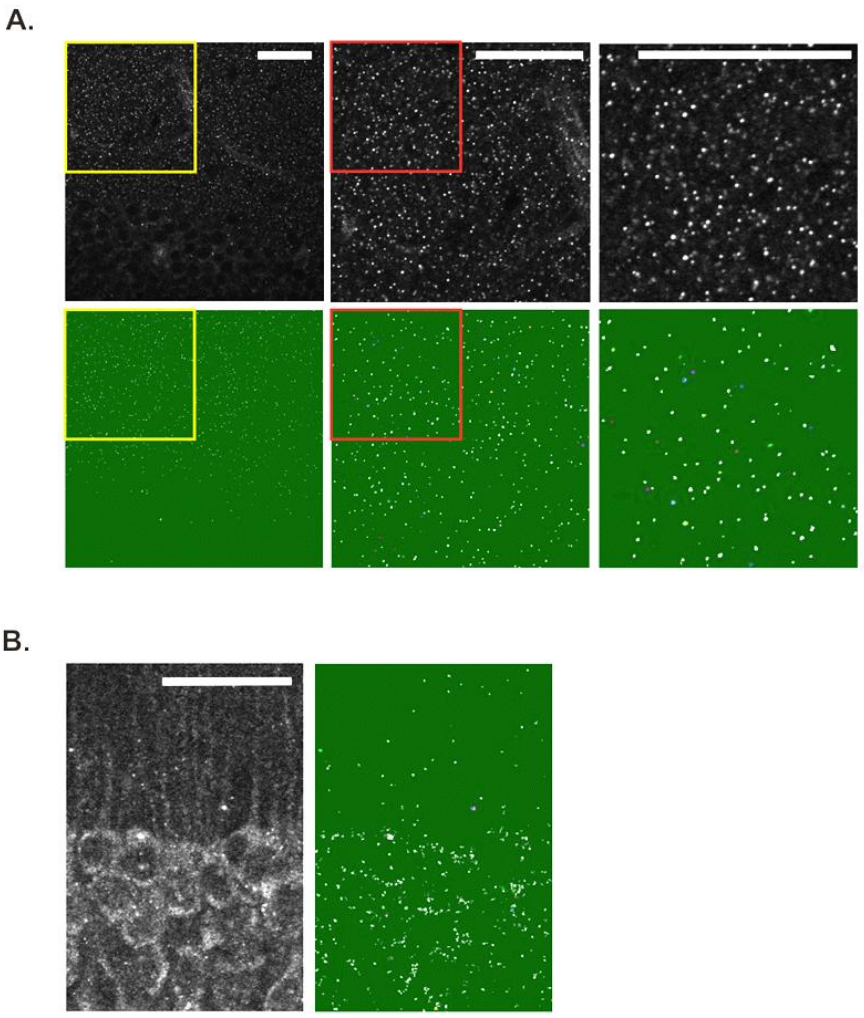

Figure 2.3 – FociPicker3D successfully identifies immunopositive punctae if images lack diffuse staining

A. Series of images demonstrating good correlation between immunopositive punctae for α3 staining in the dentate gyrus (top) and punctae identified by FociPicker3D (bottom). Images on the left represent the entire field of view (63x zoom), middle images represent a zoom into the yellow box, images on the right are zoomed into the red box from middle images. With appropriate threshold settings, FociPicker3D identifies the majority of immunopositive punctae in the image, and rarely picks up background fluorescence as false positives. Scale bars (white) are 30 µm.

B. Representative example for α5 immunostaining in the CA1 region of the hippocampus (raw image on left, Foci Picker result on the right). The program struggles to identify true punctae in images that contain diffuse as well as punctate staining. Some regions of diffuse staining wrongly identified as regions of many small individual punctae.

2.5. HEK293 cell culture and electrophysiology

2.5.1. HEK293 cell culture

HEK293 cells were maintained on 10 cm plates (Greiner-Bio-One GmbH, Frickenhausen, Germany) in a culture medium comprising Dulbecco's modified Eagle medium supplemented with 10% v/v fetal bovine serum, 100 µg/ml streptomycin, 100 U/ml penicillin G and 2 mM glutamine (all from Gibco, Invitrogen Ltd.). Plates were incubated at 37 ^{o}C in 95% air/5% CO_2 (BOC Healthcare, Manchester, UK). Cultures were passaged at approximately 70-80% confluency and plated at appropriate dilution either on to 10 cm plates for maintenance, poly-L-lysine (100 µg/mg)-coated 18 mm coverslips (VWR international) for electrophysiology, or 6 cm plates (Nunclon-Δ Surface, Thermo Fisher Scientific Inc.) for biochemistry.

For passage, cells were washed with 5 ml Hank's balanced salt solution (HBSS), and detached by trypsinisation with 2 ml 0.05% w/v trypsin-EDTA (Gibco). Cells were resuspended in 10 ml culture medium, which quenches the trypsin, and then pelleted by centrifugation at 1000 r.p.m. for 5 min. Supernatant was removed, and the cell pellet re-suspended in 5 ml culture medium by trituration with a fire-polished glass Pasteur pipette (VWR International), to ensure a single-cell suspension before plating.

2.5.2. HEK293 cell transfection

For electrophysiology, HEK293 cells were transfected by a calcium phosphate precipitation method. After plating on coverslips, cells were exposed to a mixture comprising 1 µg of each cDNA expression vector encoding the required $GABA_A$ receptor subunits (murine α, β, γ or δ cDNA housed in a pRK5 plasmid

vector), 1 µg of cDNA expression vector encoding enhanced green-fluorescent protein (eGFP), 20 µl 340 mM $CaCl_2$ and 24 µl 2 x HBSS (280 mM NaCl, 2.8 mM Na_2HPO_4, 50 mM HEPES, pH 7.2). Cells were subjected to electrophysiology 18-48 h later.

For Western blotting, cells were prepared as described in *Section 2.5.1.*, except that after trypsinisation, cells were pelleted and washed by resuspending in 10 ml OptiMEM I (Gibco) and again sedimented by centrifugation. This cell pellet was resuspended in 400 µl OptiMEM I per transfection and transferred to BioRad 0.4 cm electrode gap cuvettes containing the appropriate cDNA vector mixture (2 µg per $GABA_A$ receptor subunit). Cells were then electroporated using a BioRad gene pulser II electroporator, and recovered with 0.5 ml culture medium before plating on poly-L-lysine-coated 6 cm culture dishes. Protein was harvested from cells 2-3 days post-electroporation.

2.5.3. HEK293 cell electrophysiology

Coverslips containing transfected HEK293 cells were transferred to a recording chamber, and continuously perfused at RT with Krebs solution, containing (mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.5 glucose, 11 HEPES and 5 $CaCl_2$ (pH 7.4). Whole-cell recordings were undertaken from transfected (GFP-positive) HEK293 cells located using epifluorescence optics (Nikon Eclipse E600FN, Nikon Instruments Europe B.V. Surrey, UK). Membrane currents were recorded using a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, California, USA) in the voltage-clamp configuration (holding potential of -20 mV). Currents were filtered at 4 kHz (8^{th} order Bessel, -48 dB/octave) and digitized at 50 kHz (Digidata 1322A, Molecular Devices), and displayed using Clampex software (version 8.2, Molecular Devices). Patch pipettes (4-5 MΩ) were filled with an internal solution containing (mM): 120 KCl, 1 $MgCl_2$, 11 EGTA, 10 HEPES, 1 $CaCl_2$, 4 ATP (pH 7.2). The osmolarity of the internal solution was 300 ± 20 miliOsmoles/litre (mOsm/l), measured using a vapour pressure osmometer

(Wescor Inc, Utah, USA). Responses of cells to brief (2-4 s) applications of GABA, alone or in combination with other drugs, were recorded using the gap-free recording mode in Clampex. Substance applications were made using a Y-tube (*Fig. 2.4*), and with 2 min recoveries between applications.

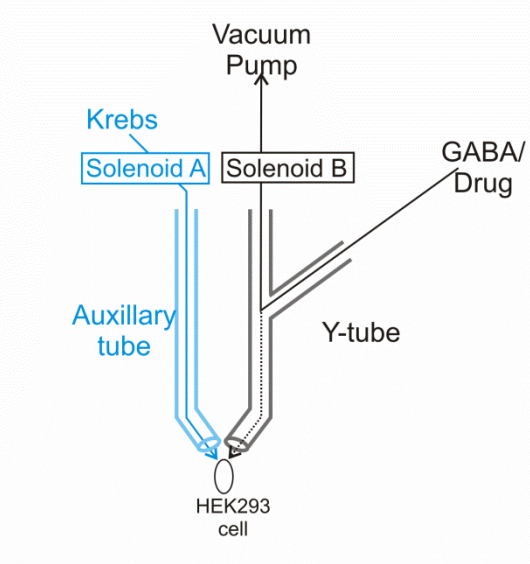

Figure 2.4 – Schematic representation of the Y-tube

Auxiliary/wash and Y-tube/drug-application tubes are arranged as depicted. Between drug applications, solenoids are open – allowing Krebs to flow over the cell, and drug to flow to waste under vacuum pressure. During applications, solenoids close, allowing drug to be applied to the cell (dotted line) in the absence of washing Krebs.

Responses were measured by comparing peak amplitude to the baseline holding current in Clampfit software (version 10.2, Molecular Devices). To monitor for run-up or run-down of GABA responses, a defined concentration of GABA was applied at regular intervals to the same cell. Between applications, series resistance (R_s) was monitored. Experiments were terminated if R_s changed by more than 20%. GABA concentration-response curves were constructed by plotting mean peak responses against the GABA concentration, and fitting these data using the Hill equation (equation 1).

Equation 1

$$I_{[GABA]} = I_{max} * ([GABA]^n / (EC_{50}^n + [GABA]^n))$$

Where $I_{[GABA]}$ is the peak current activated by GABA at a concentration [GABA], I_{max} is the maximal current response to GABA, EC_{50} is the concentration of GABA producing a current response 50% of the maximal response, and n is the Hill coefficient.

GABA concentration-response curves (*Fig. 3.1*) demonstrate that 0.5 µM GABA corresponds to an EC_{15} concentration (producing 15% of the maximal response) for both $\alpha2^{WT}\beta3\gamma2s$ and $\alpha2^{Q241M}\beta3\gamma2s$ combinations. This concentration was selected for determining potentiation by THDOC and/or diazepam. The peak response to 0.5 µM GABA + drug was divided by that produced by a preceding control application of 0.5 µM GABA alone. The diazepam potentiation curve was fit with a modified version of equation 1, to include the pedestal (equation 2).

Equation 2

$$P_{[dzp]} = P_{min} + (P_{max} - P_{min}) * ([dzp]^n / (EC_{50}^n + [dzp]^n))$$

Where $P_{[dzp]}$ is the peak potentiation activated by 0.5 µM GABA plus a particular concentration of diazepam ([dzp]), P_{max} is the maximal potentiation achieved with diazepam, EC_{50} is the concentration of diazepam producing 50% of the maximal potentiation, and n is the Hill coefficient.

2.6. Brain slice electrophysiology

2.6.1. Preparation of slices

Coronal slices (350 µm) were obtained from wild-type and transgenic mice using a Leica VT1200s vibroslicer (Leica Microsytems GmBH, Wetzlar, Germany). Sections of nucleus accumbens were cut in an ice-cold artificial cerebrospinal fluid (aCSF) comprising (mM): 85 NaCl, 2.5 KCl, 1.25 $Na_2H_2PO_4$, 26 $NaHCO_3$, 1 $CaCl_2$, 4 $MgCl_2$, 25 glucose, 75 sucrose and 2 kynurenic acid. Hippocampal slices were cut in ice-cold aCSF comprising (mM): 130 K-gluconate, 15 KCl, 0.05 EGTA, 20 HEPES, 4 Na-pyruvate, 25 glucose and 2 kynurenic acid, pH 7.4). Slices were transferred to a holding chamber in a water bath set at 37 ^oC, whilst the solution was slowly exchanged over 1 h to a

recording aCSF of composition (mM): 125 NaCl, 2.5 KCl, 1.25 $Na_2H_2PO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$, 25 glucose. Slices were then maintained in the holding chamber at RT. All aCSF solutions were continuously bubbled with 95%O_2/5%CO_2 (BOC Healthcare).

2.6.2. Whole-cell patch clamp recording

Whole-cell recordings were undertaken at RT from single neurons located using infra-red optics (Nikon Eclipse E600FN, Nikon Instruments Europe B.V. Surrey, UK) fitted with a Basler SLA750-60fm Camera (Basler Vision Technologies, Ahrensburg, Germany). Membrane currents were recorded as described for HEK293 cells (*Section 2.5.3*), using patch pipettes (3.8-4.5 MΩ) filled with an internal solution containing (mM): 140 CsCl, 2 NaCl, 10 HEPES, 5 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, 2 Na-ATP, and 2 QX-314 Bromide (Ascent Biochemicals, Abcam plc, Cambridge, UK). Solution osmolarity was adjusted with sucrose, usually 5 mM for hippocampal slices (approximately 305 mOsm/l) and 12.5 mM for NAcc slices (approx. 315 mOsm/l).

Slices were continuously perfused with recording aCSF supplemented with kynurenic acid, to isolate GABAergic events. The neurosteroid, THDOC was dissolved as a 10 mM stock solution in dimethyl sulphoxide (DMSO), and diazepam was dissolved as a 100 mM stock in DMSO. These drugs were diluted to the appropriate final concentration in the recording aCSF, and applied to cells in the bath after a period of stable control recording. Drugs were allowed to equilibrate in the bath for at least 5 min; control or 'mock' recordings were performed to confirm that there are no changes in synaptic currents due to the DMSO vehicle, or washout of intracellular contents independent of drug (i.e. cells were challenged with 0.01% (v/v) DMSO[1], or simply held in control aCSF

[1] Equivalent to the highest % DMSO applied to cells (when administering 100 nM THDOC)

for an equivalent duration to experiments where drug was applied)[2]. Experiments were completed by the bath application of 20 µM (-)-bicuculline-methiodide: to confirm that all events were GABAergic, and to allow any GABA-mediated tonic currents to be measured. Recordings were made in 2 min epochs, between which R_s was monitored, and experiments were terminated if R_s changed by more than 20%.

2.6.3. Analysis of spontaneous inhibitory post-synaptic currents (sIPSCs)

To determine the frequency of sIPSC events in a given recording, all visible events in an epoch were identified using MiniAnalysis software (Synaptosoft Inc., Fort Lee, New Jersey, USA), and inter-event interval times determined. Not all events are appropriate for further analysis because, for example, their decay is contaminated with the presence of another sIPSC event. Only clean, uncontaminated events were selected for decay fitting in MiniAnalysis, using either a mono- or bi-exponential function. The best fit was determined by eye and also by the increasing value of the parameter R^2. In order to combine analysis of mono- and bi-exponentially decaying events, decay times were transformed to a weighted decay time, τ_w, according to *equation 3*.

Equation 3

$$\tau_w = (A_1.\tau_1 + A_2.\tau_2) / (A_1 + A_2);$$

Where τ_1 and τ_2 are the time constants for a biexponential decay, and A_1 and A_2 are the relative amplitude contributions of τ_1 and τ_2, respectively. For monoexponential decay, terms A_2 and τ_2 are absent (i.e. $\tau_w = \tau_1$).

For each IPSC event, four parameters were measured: rise time, amplitude, τ_w and area (charge transfer). A minimum of 100 events per condition (control vs.

[2] Both controls gave equivalent results so have been combined

drug) were fitted, and average sIPSC parameters were determined. The results are expressed as a percentage change for the test epoch relative to the control epoch in that cell; i.e. +30% and -30% would represent a 30% increase and decrease, respectively, for a particular parameter.

2.6.4. Analysis of tonic GABA currents

Tonic currents are revealed by observing a reduced holding current and root mean square (r.m.s.) current noise on application of the competitive $GABA_A$-receptor antagonist, 20 µM bicuculline. Changes in holding current were often small (less than 10 pA) and changes in r.m.s. noise proved to be a more robust and reliable measure of changes in tonic current in this study. WinEDR (version 3.1) software (John Dempster, University of Strathclyde, Glasgow, UK) was employed to measure r.m.s. noise over 100 ms epochs of a recording. The presence of any synaptic events in a single 100 ms epoch will increase the r.m.s. value, and so such epochs must be excluded from analysis. It is possible to do this manually by scrolling through records and marking those contaminated epochs for exclusion, but we used an automated procedure. Briefly, Microsoft Excel was employed to compare the r.m.s. value of each 100 ms epoch to a user-defined threshold, and automatically exclude those with values above the threshold as ‘contaminated’. The threshold is defined as a proportion of the median r.m.s. current over a local 5 s window of recording (50 x 100 ms epochs). This ‘thresholding approach’ was validated by comparison with a small section of data (approx. 60 s) where contaminated 100 ms epochs were excluded manually. This threshold was then applied to the remainder of the data from that cell. For both tonic and synaptic current analyses, time courses indicated that the full effect of drug was achieved within 5 min, and so data report the changes in r.m.s. noise or IPSC parameters after at least 5 min equilibration of drug.

2.7. Behavioural Analyses

2.7.1. Animal handling and drug administration

All experimental procedures were performed between 07:30h and 12:30h. Animals were transferred from their housing facility to the experimental room 1 hour before testing commenced. During this habituation time, mice were housed individually in cages, with access to ample food and water. All animals were used in a single behavioural test, and were experimentally naïve. Before behavioural testing, mice were subjected only to routine handling for husbandry and ear notching.

Where drugs were administered, mice received substances by intraperitoneal injection (27 gauge ½" needle, 1 ml syringe) at a volume of 10 ml per kg body weight. The timings of injection were either 15 min (THDOC, sodium pentobarbital) or 30 min (diazepam) before behavioural testing. THDOC was dissolved in 0.9% w/v NaCl containing 25% w/v 2-hydroxypropyl-β-cyclodextrin vehicle. Diazepam was prepared in 0.9% w/v NaCl containing 3.5 % v/v DMSO vehicle. Sodium pentobarbital was dissolved directly in 0.9% w/v NaCl. Dose-ranges and timings for injection were determined by examining those used elsewhere in the literature and by performing preliminary experiments with these mice (e.g. timecourses for the action of THDOC elsewhere (Crawley *et al.*, 1986) and here (*Fig. 2.8*) indicate that anxiolytic and motor-impairing effects peak within 15 min post intraperitoneal injection).

2.7.2. Elevated plus maze

The elevated plus maze is a standard screen for anxiety-like behaviour, validated with anxiolytic drugs (Pellow *et al.*, 1985; Pellow & File, 1986). The

maze comprised a horizontal black Perspex cross, with a central square of 50 x 50 mm, from which extend four arms, each to a length of 300 mm (*Fig. 2.5*). Two 'closed' arms were surrounded by black Perspex walls (height 150 mm), and the other two 'open' arms had only a raised edge (approx. 2 mm). The maze was elevated 500 mm above the floor of the experimental room, and the illumination across the arms was uniform and low intensity (approximately 25 lux, equivalent to 25 lumen per square metre).

Mice were placed in the centre of the maze, initially facing one of the closed arms. Their movements within the maze over a 5 min trial were monitored with a Sony Handycam (HDR-CX-190E) camcorder mounted 1.5 m directly above the maze. Between tests, the maze was wiped down with distilled water, and allowed to dry. The number of entries into each arm and the time spent in each arm was scored blind to genotype. A mouse was defined as having entered an arm when all four paws were within the boundary of the arm. The percentage time in an arm is expressed as (time in arm / total time of experiment) x 100.

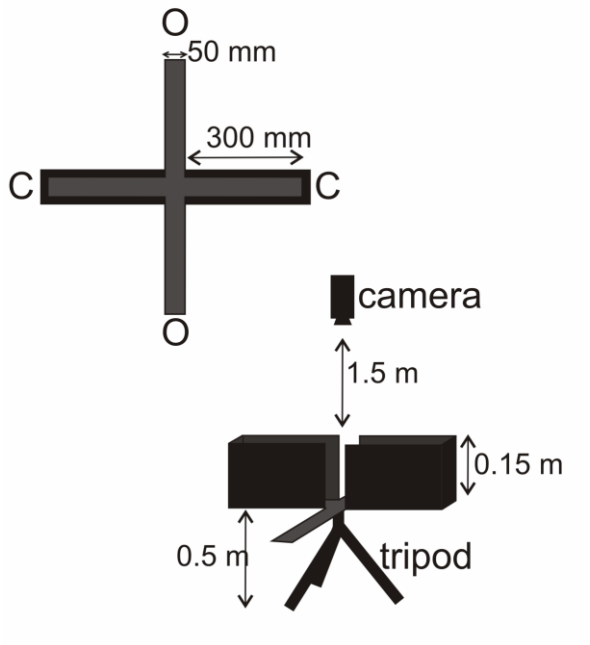

Figure 2.5 – The elevated plus maze

The maze is depicted from plan (top) and side elevation views (bottom). The closed arms (C) have black Perspex walls 15 cm high, whilst open arms (O) have only a slight raised edge. The maze is elevated by mounting on a tripod, and behaviour on the maze is scored from a video captured by a camera mounted above the maze.

For examining baseline anxiety levels, three mice were tested each day – one of each genotype – in a randomized order. When examining the anxiolytic effects of drugs, mice were tested in pairs (one hom and one wt), where the drug dose received, and the order in which mice were tested (wt first vs. hom first) had been randomised. Videos were scored blind to both the genotype and drug administered.

Note that whilst some investigators include a series of ethological measures of 'risk assessment' in their anxiety studies (Rodgers & Johnson, 1995, 1998), we have not included this analysis here for a number of reasons. There is a degree of subjectivity and ambiguity when scoring particular risk assessment behaviours, not aided by our study using a black mouse, which is filmed on a black background under dim lighting. Moreover, it is difficult to say what a reduction in risk assessment behaviour actually represents: reduced anxiety, increased anxiety or activity effects (Blanchard *et al.*, 1990). We have therefore focussed on spatiotemporal scores for anxiety (percentage time on open arms etc.), which respond more consistently to a range of anxiolytics than ethological measures (Rodgers & Johnson, 1998).

2.7.3. Light-Dark Box

The light-dark box is a standard screen for anxiety-like behaviour, with validity for anxiolytic drugs (Crawley & Goodwin, 1980). The box dimensions were 20 x 45 cm, which were divided into a light zone (850 lx) and a dark zone (50 lx) connected by a small doorway. The light zone has an area twice that of the dark zone and the floors of each zone are marked with a grid to allow scoring of activity (*Fig. 2.6*). A mouse was placed initially in the light zone, facing away from the doorway, and was then allowed to freely explore the box for 10 min. Three mice did not move for at least the first 5 min of the test, and have been excluded from the analysis. Between tests, the box was wiped clean with distilled water, and allowed to dry.

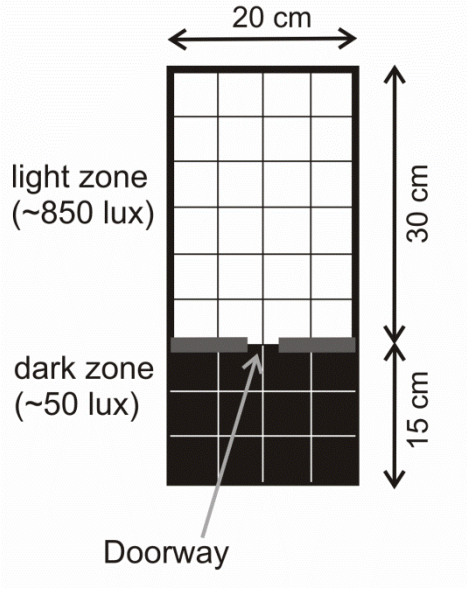

Figure 2.6 – The light-dark box

Plan view is shown. The light zone consists of white Perspex floor and walls (25 cm high), and the dark zone of black Perspex. Zones are connected by a small doorway at floor level. The floor of each zone is divided into equally-sized squares (5 x 5 cm) to allow activity to be scored (see main text).

To compare the effects of genotype on baseline anxiety, four mice were tested each day (two wt and two hom) – in a randomised order. To compare the effect of drug treatments, mice were tested in pairs – one wt and one hom; the order in which they were tested (wt first, or hom first), and the drug dose received, were randomised.

A video record of the test was scored blind to the genotype and any drug treatment of the mouse. Scoring assessed the time to leave the light zone, the time spent in each zone, the number of transitions between each zone. In addition, the exploratory activity within each zone was measured by counting the number of grid-lines crossed, then correcting for the amount of time spent in each zone (i.e. activity is expressed as line crossings per second).

2.7.4. Tail-suspension test

The tail suspension test is a standard screen for depression, with validity for anti-depressant drugs (Steru *et al.*, 1985; Thierry *et al.*, 1986). Mice were

suspended by the tail with 19 mm wide PVC insulation tape (Powerlink Plus+, Lancashire, UK) from a metal bar, raised 55 cm above the benchtop (see *Fig. 2.7*). Because of the tendency of the C57BL/6J strain to climb their tail in this test, the 'climbstopper' approach described by Can *et al.* (2012) was used – i.e. a small plastic cylinder (4 cm long, 1 cm internal diameter, approximately 1 gram weight) was placed over the base of the tail before attaching the sticky tape. With the equipment depicted in *Fig. 2.7*, four mice can be tested simultaneously. The mice cannot observe or interact with each other due to the opaque dividers between compartments. Two wt and two hom animals were tested together, with the drug dose applied, and compartment in which they were placed, determined in a counterbalanced, randomised design. The behaviour of mice was filmed over a 6 min period.

The scoring was performed blind to the genotype and drug dose applied, using an Xnote stopwatch (dnSoft Research Group) to count the cumulative time for which the mouse is immobile over the six minutes of the test (Can *et al.*, 2012). Small movements of front paws, without hind leg movements, are not counted as escape-related mobility; similarly pendulum-like motion, resulting from momentum gathered in previous bouts of motion, is not counted as mobility. An increased immobility can represent a depressed phenotype, although care must be taken to ensure this is not a sedative effect. Conversely a decrease in immobility is thought to represent a reduction in depression, again taking care to ensure this is not a stimulant effect.

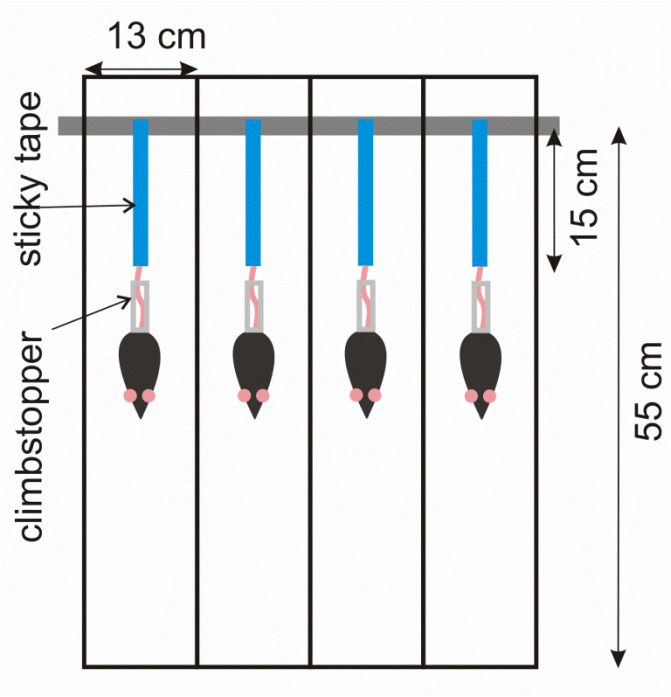

Figure 2.7 – Tail suspension test equipment

A white box 60 cm in height is divided into four compartments, 13 cm wide and 11 cm deep. Mice are suspended from an aluminium bar (grey), elevated 55 cm above the bench top, using sticky tape (blue). Before attaching to the bar, tails are also fitted with a climbstopper (*see main text*). Dividers between compartments (black lines) prevent mice from observing one-another during the test.

2.7.5. Rotarod

The rotarod test is a standard screen for motor coordination defects induced by genetic mutation or drug treatment of rodents (Dunham & Miya, 1957; Jones & Roberts, 1968). Mice were placed on a five-station rotarod treadmill apparatus (Med Associates Inc., Vermont, USA) with the rod rotating at an initial speed of 4 r.p.m. The rod was then programmed to accelerate uniformly over a period of 5 min up to a final speed of 40 r.p.m. If a mouse failed the test by falling off the rod before the full 5 min trial, it was removed from the apparatus until the next trial. The time at which a mouse fell from the rotarod was recorded by the MedAssociates software, triggered by a photocell beam break. In some instances, a mouse will hold on to the rod and rotate with it – termed a 'passive rotation' – this is also considered a failure; the time at which this occurred was noted manually. If a mouse completed the full test, the 'time to failure' was simply set as the duration of the test (300 s).

The performance of each mouse on his first encounter on the rod (green bar, *Fig. 2.8*) was used as a measure of the baseline motor coordination. Each mouse was subjected to further training on the rotarod, such that a consistent performance was attained before the effect of drug was assessed. Training consisted of 5 trials on the rotarod per day, with a 10 min break between trials (*Fig. 2.8*); training was carried out over four consecutive days. On the fifth day, each mouse mice was injected intraperitoneally with THDOC (or vehicle) 15 min before his first exposure to the rod (arrow, *Fig. 2.8*). The drug dose administered to each mouse was pre-determined by a counterbalanced randomisation procedure.

To assess any impairment of motor coordination caused by a drug, the performance of each mouse under drugged conditions was expressed relative to that mouse's average performance on the rod on the preceding day (i.e. average of blue trials, *Fig. 2.8*). Each mouse was tested five times under the influence of drug, once every 15 min, starting from 15 min after injection. The recovery from any motor impairment induced by THDOC was fast (*Fig. 2.8B*), therefore the effect of THDOC was expressed as the time to failure on the first trial after injection, normalised to the trials on day 4 of training.

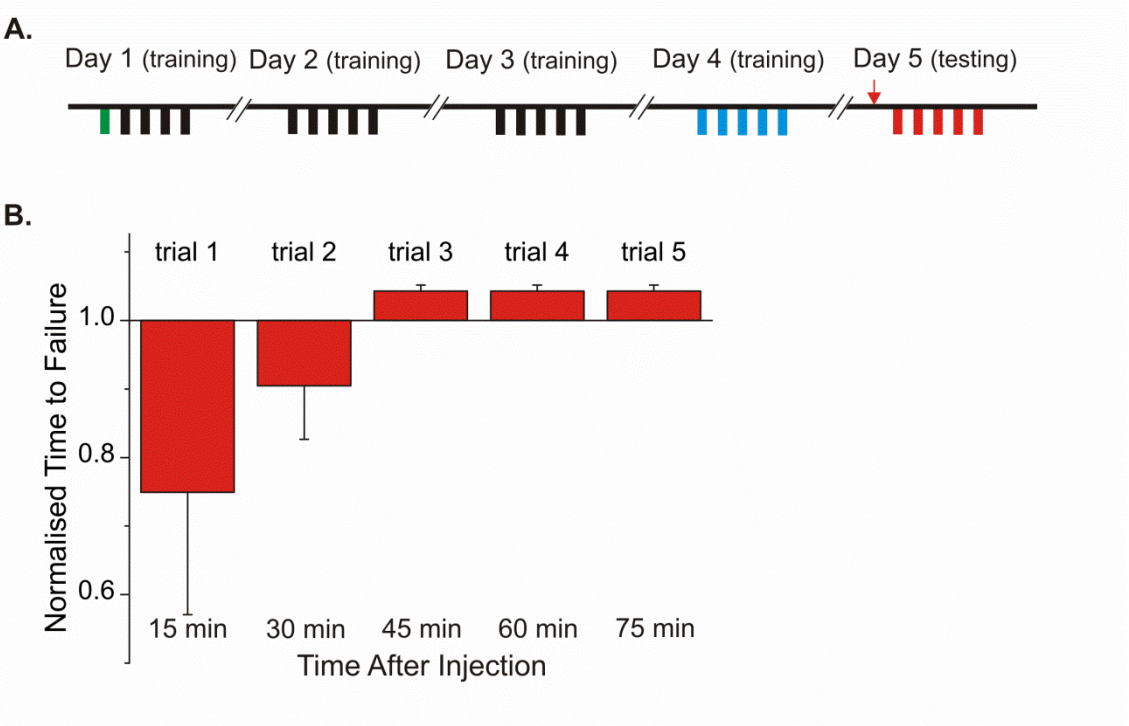

Figure 2.8 – Rotarod protocol

A. The rotarod protocol; each downward bar represents a 5 min trial on the accelerating rotarod, mice were rested 10 min between exposures. The first exposure (green) gives a measure of baseline motor coordination. On the testing day, mice were injected (red arrow) 15 min before exposure to the rotarod. The performance under the influence of drug (red) is normalised to the average performance under non-drugged conditions after training (blue).

B. The motor-impairing effects of 20 mg/kg THDOC were short-lived, and most clearly apparent in the first trial (15 min after injection); recovery to baseline was achieved by trial 3 (45 min after injection). Comparisons therefore focus on the trial-1 effects of THDOC.

2.8. Statistics

All data are presented as mean ± standard error of the mean (s.e.m.). Graphical representations of data were plotted using Microcal OriginPro (version 8.5;

OriginLab Corporation, Northampton, MA, USA), and figures assembled using CorelDraw (version X4; Corel UK Ltd., Maidenhead, Berks, UK).

For most of the data analyses, parametric comparisons (analysis of variance (ANOVA) and t-test) were first considered for use. These tests assume that the data are normally distributed, and that the variances of the compared groups do not differ. Tests were performed to verify that these constraints were met (e.g. Bartlett's test to compare standard deviations of data sets). Where the data did not satisfy these conditions, they were variously transformed (e.g. logarithmic transform), and the criteria were reassessed before proceeding with any statistical comparison. In instances where transformation was unable to produce data satisfying the conditions for parametric comparison, data were instead subjected to non-parametric analysis using the Kruskal-Wallis test. If, by applying the Kruskal-Wallis test to all the data indicated a significant variation between discrete groups, individual pairwise comparisons were performed using InVivoStat (British Association for Psychopharmacology, Cambridge, UK), which recommends the Behrens-Fisher test in preference to the Mann-Witney (although both tests generally gave equivalent results), primarily because it allows for non-normal distributions (e.g. skewed) and heteroscedascity of the data (Munzel & Hothorn, 2001). Furthermore, when comparing the analysis of known data-sets, the Behrens-Fisher test gave the most robust statistics (Steland *et al.*, 2011).

Parametric one-way ANOVA and t-test comparisons were performed using InStat 3 (GraphPad Software, La Jolla, California, USA), with all-pairwise comparisons performed, and adjusted for multiple comparisons with the method of Bonferroni. For the time-course analysis of light-dark box results, repeated measures ANOVA was performed, with individual pairwise comparisons performed for each time bin relative to bin 1, using Dunnett's test.

Parametric two-way ANOVA were performed using InVivoStat to report the Fisher's least-significant difference (LSD) p-values from planned individual pairwise comparisons (i.e. not every individual pairwise comparison was made,

but only a selected proportion were chosen, on the basis of scientific relevance, before the experiment commenced). Even though the probability of a type I error (inappropriate rejection of a null hypothesis) was reduced by using only planned comparisons, the risk of such an error is high when considering the large numbers of comparisons made within each experiment (as high as 25). We therefore adjusted the LSD p-values using the method of Benjamini and Hochberg (Benjamini *et al.*, 2001). This is a means to control the 'false discovery rate', which limits the proportion of type I errors to 5%. When considering such a large number of comparisons, we favour this approach over that of Bonferroni (which reduces the significance level, alpha, in proportion to the number of comparisons made). The Bonferroni method controls the family-wise error rate, ensuring a 5% chance of a single type I error from all comparisons made, but this increased robustness comes at the price of a high type II error rate (failing to reject null hypotheses that should be rejected).

Chapter 3: Creating a transgenic mouse with disrupted neurosteroid potentiation at the $GABA_A$ receptor α2 subunit

3.1. Introduction

As discussed in the *Section 1.2.2*, observations that $GABA_A$ receptor Cl^- conductance is modulated in a stereo-selective and biphasic manner by neuroactive steroids led to proposals that there are two specific neurosteroid binding sites on the $GABA_A$ receptor: an "activation site" and a "potentiation site" (Harrison & Simmonds, 1984; Harrison *et al.*, 1987; Puia *et al.*, 1990; Paul & Purdy, 1992). In defining these neurosteroid binding sites, Hosie *et al.* (2006) demonstrated that hydrophobic substitution of a critical $\alpha1^{Q241}$ residue was sufficient to selectively disrupt neurosteroid potentiation. For example, mutation $\alpha1^{Q241M}$ eliminated potentiation by allopregnanolone, with minimal effects on receptor activation by GABA and modulation by other allosteric ligands (barbiturates, benzodiazepines). This work also demonstrated that the full effect of neurosteroid binding to the activation site is only achieved by concomitant binding of neurosteroid molecules to the potentiation site (i.e. mutation $\alpha1^{Q241M}$ disrupts both potentiation and direct activation by neurosteroids). Hydrophobic substitution of the conserved $\alpha1^{Q241}$ residue has been extended to the other five α subunit isoforms, in every case the substitution disrupted potentiation by neurosteroids (Hosie *et al.*, 2009).

This discovery provided a unique opportunity to dissect the roles of the various α subunit isoforms in the physiological and pharmacological functions of neurosteroid molecules. By creating a *knock-in* transgenic mouse in which genomic DNA encoding specific α subunit has been replaced with a Q-to-M mutant copy of the receptor, one can assess the consequences of losing neurosteroid modulation of that receptor subtype on synaptic inhibition and mouse behaviour. Whilst such questions could be addressed with α-subunit knock-out mice, this approach is often hampered by lethality (e.g. γ2 knock-out

mice are not viable, restricting study to heterozygous knock-outs (Gunther *et al.*, 1995)) or results in compensatory changes in expression of other subunits (e.g. α1-/- mice show increased expression of α2 and α3 subunits (Kralic *et al.*, 2002)). With the knock-in approach, mutant receptors should be expressed and respond to GABA as if wild-type (Hosie *et al.*, 2006). Phenotypic changes in the mouse would therefore probably result from lack of neurosteroid function at this receptor, rather than a compensatory alteration in expression. However, caution should be exercised when making such an assumption: our model would involve losing response to endogenous molecules, and the effects of losing any such modulation under baseline conditions may be significant, and theoretically could result in compensatory changes. The latter half of this chapter deals with verifying a lack of compensation in our transgenic model.

In this body of work, the Q-to-M substitution has been introduced in the α2 subunit – i.e. knock-in mice harbouring the mutation $\alpha2^{Q241M}$ have been generated. Whilst the $\alpha2^{Q241M}$ mutation has been shown to disrupt potentiation by neurosteroid (Hosie *et al.*, 2009), further criteria must be met by the mutation for it to be appropriate for the mouse model; specifically that GABA sensitivity is preserved, and subunit expression should be unperturbed. We would also expect benzodiazepine potentiation to be unaffected by the mutation. These features have been verified in this study by characterising responses of α2β3γ2 $GABA_A$ receptors expressed in HEK293 cells. This approach was also used to probe the effects of concurrent modulation of these receptors with neurosteroids and benzodiazepines.

The α2 isoform was selected as the first candidate for generating a knock-in mouse because of its strong links with the mammalian anxiety circuitry (*see Section 1.3.2*), and we predict that neurosteroid anxiolysis will occur via α2-type $GABA_A$ receptors. This hypothesis is addressed in later chapters, but firstly screens must be performed to ensure a lack of underlying compensatory changes that could account for any behavioural effects of the mutation. In this study, assays to measure $GABA_A$ receptor expression at the protein level have

been chosen (Western blot and quantitative immunofluorescence), as these are the proteins that ultimately mediate inhibitory transmission. $GABA_A$ receptor subunits α1-α5 were selected as the most likely candidates to be up- or down-regulated in response to the mutation, and so subjected to quantitation. Assays were focussed on four brain areas that express $GABA_A$ receptor α2 subunits – cortex, hippocampus, cerebellum and nucleus accumbens (Laurie *et al.*, 1992a; Wisden *et al.*, 1992; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002).

3.2. Results

3.2.1. Confirming that $\alpha2^{Q241M}$ has no effect on GABA and diazepam sensitivity of αβγ receptors

The effects of $\alpha2^{Q241M}$ on receptor function were examined by characterising α2-containing $GABA_A$ receptors expressed in HEK293 cells. This cell line has been used widely as a platform for expression and study of $GABA_A$ receptor properties. Because of their non-neuronal origin, these cells do not express endogenous $GABA_A$ receptors (although there has been evidence for some low level expression of β3, γ3 and ε subunits (Thomas & Smart, 2005)), allowing one to control the composition of $GABA_A$ receptor heteropentamers under study. For electrophysiology, cells were transfected with expression plasmids for each of the subunits in the combination α2β3γ2S, together with an expression plasmid for eGFP (see *Section 2.5*). This combination of receptor subunits is thought to be representative of native α2-type $GABA_A$ receptors *in vivo* (Benke *et al.*, 1994; McKernan & Whiting, 1996). The eGFP allows identification of transfected cells, by their green fluorescence, for study by whole-cell patch clamp electrophysiology.

Peak current responses of wild-type (α2β3γ2S) and mutant ($\alpha2^{Q241M}$β3γ2S) receptors were measured in response to brief (2-4 s) applications of GABA at concentrations 0.01-1000 μM (*Fig. 3.1 C*). GABA concentration-response data were collected for each cell, and fitted with the Hill equation (*Equation 1, Section 2.5.3*). Mean Hill-fit parameters, EC_{50} (the concentration of GABA eliciting a 50% maximal response) and n (Hill coefficient), for wild-type and mutant receptor combinations are not different (*Table 3.1*). This is consistent with previous observations that the $\alpha2^{Q241M}$ mutation is without effect on the GABA sensitivity of the α2β3γ2S receptor (Hosie *et al.*, 2009). The lack of effect of the $\alpha2^{Q241M}$ mutation on the GABA sensitivity can also be appreciated by noting the good superimposition of the mean GABA concentration-response curves for these receptors (*Fig. 3.1 A*).

To confirm that the $\alpha2^{Q241M}$ mutation did not affect benzodiazepine modulation, concentration-potentiation curves for diazepam potentiation of wild-type and mutant receptors were constructed, as outlined in *Section 2.5.3*. Briefly, peak current responses of a transfected cell to 0.5 μM GABA (EC_{15} response) co-applied with varying concentrations of diazepam were normalised to peak current responses to 0.5 μM GABA alone within that cell. As above, concentration-potentiation curves were constructed for each cell, this time using a modified Hill equation, to include a pedestal (*Equation 2, Section 2.5.3*). The mean fit parameters, summarised in *Table 3.1*, are very similar. There is a small but significant increase in the Hill coefficient for mutant vs. wild-type receptors, but when mean data are plotted together (*Fig. 3.1 B*) the concentration-potentiation curves are superimposed. The diazepam sensitivity of the α2β3γ2S receptor combination is therefore unchanged by $\alpha2^{Q241M}$ mutation, consistent with previous work characterising this mutation on α1 (Hosie *et al.*, 2006). These data demonstrate that this substitution specifically abolishes potentiation by neurosteroids.

Dose relationship		**wild-type**	**Q241M**
GABA concentration-response	**EC_{50} (μM)**	3.0 ± 0.8	3.2 ± 0.4
	n	1.2 ± 0.2	1.2 ± 0.1
Diazepam concentration-potentiation	**P_{min}**	0.9 ± 0.04	1.0 ± 0.03
	P_{max}	2.9 ± 0.2	2.9 ± 0.3
	EC_{50} (nM)	50 ± 16	38 ± 11
	n	1.0 ± 0.1	1.7 ± 0.3*

Table 3.1 – Hill fit parameters for GABA and diazepam responses

Summary of the fits (mean ± s.e.m.) for GABA and diazepam concentration-response curves. P_{max} and P_{min} correspond to the maximum and minimum points on the diazepam potentiation curve. The Q241M mutation significantly increases the Hill coefficient (n) for diazepam potentiation (* $p < 0.05$ vs. wild-type, Mann-Witney test).

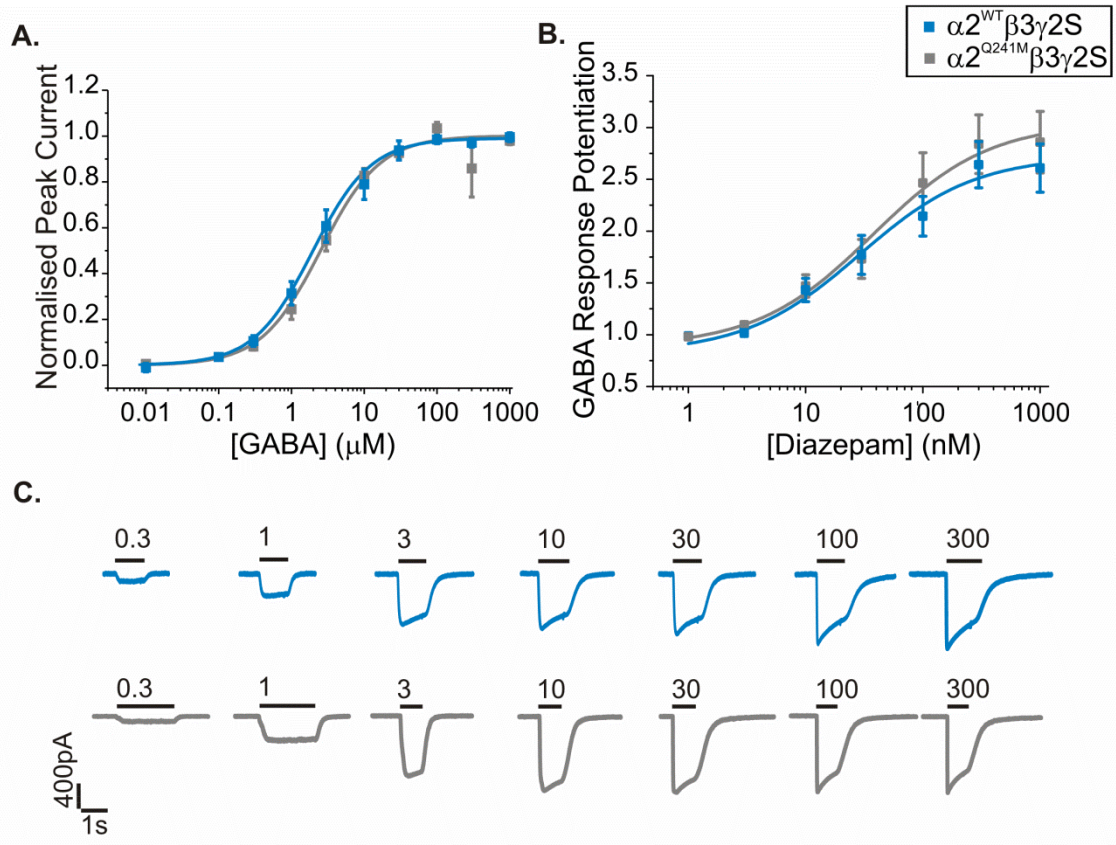

Figure 3.1 – Mutation α2^{Q241M} has no effect on the GABA or diazepam sensitivity of α2β3γ2S receptors expressed in HEK293 cells

A. and B. Concentration-response curves for wild-type (blue) and mutant (grey) α2β3γ2S receptors expressed in HEK293 cells. Peak GABA responses are normalised to the maximal response in each cell (A). Potentiation of GABA EC_{15} responses by diazepam (B) is expressed as the peak response to GABA plus diazepam relative to the response to GABA alone. Each graph represents the mean responses across 7-8 cells (error bars, s.e.m.; 3-8 data points per concentration).

C. Representative membrane currents on short applications of the indicated GABA concentrations (μM) to wild-type (blue) and mutant (grey) receptors

3.2.2. Probing the effect of $\alpha2^{Q241M}$ on responses to both THDOC and diazepam

When considering the effect of pharmacological potentiators of $GABA_A$ receptors *in vivo*, one must not forget that neurosteroid molecules are produced endogenously. This suggests that whilst the potentiating effect of diazepam on $\alpha2^{Q241M}\beta3\gamma2S$ receptors may be normal when receptors are assessed in HEK293 cells, the level of diazepam potentiation may not be 'wild-type like' when taking place on a background of endogenously-synthesised neurosteroid *in vivo*.

To assess the significance of this, transfected HEK293 cells were used to examine the effects of co-applying the neurosteroid THDOC together with diazepam, on the potentiation of an EC_{15} GABA response. A typical experiment is depicted in *Fig. 3.2 C*; briefly, peak current responses to 0.5 µM GABA were compared with those achieved by co-application of 0.5 µM GABA together with either DMSO vehicle (V), 100 nM THDOC (T) alone, 500 nM diazepam (D) alone, or both THDOC and diazepam (T + D) together. As can be seen in *Fig. 3.2 A*, in wild-type α2β3γ2S receptor-expressing cells there is no effect of vehicle, and the effects of T and D are similar, and are approximately additive, when both substances are applied together. Conversely, in mutant $\alpha2^{Q241M}\beta3\gamma2S$ receptor expressing cells (*Fig. 3.2 B*), there is no effect of THDOC alone or when co-applied with diazepam (i.e. potentiation by T + D co-application is no different to that for D alone).

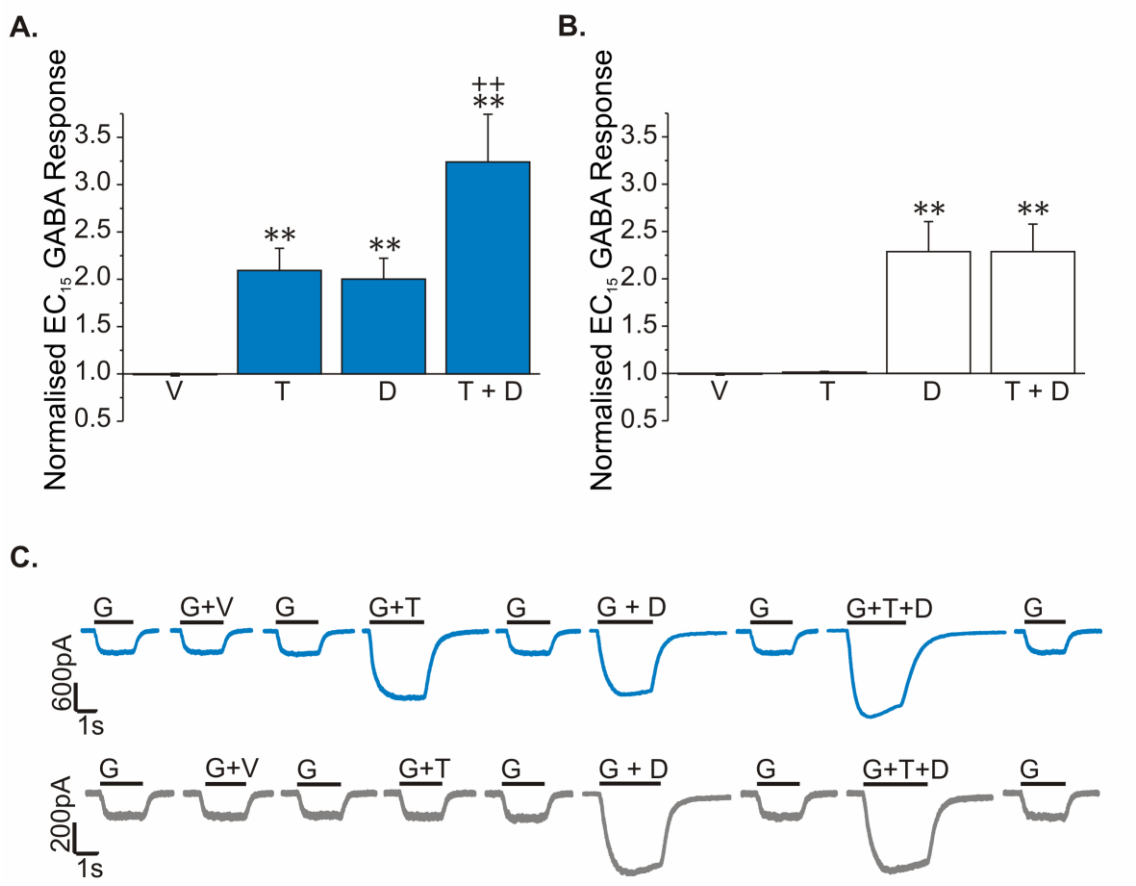

Figure 3.2 – Mutation α2^{Q241M} specifically abolishes the potentiating effect of THDOC on GABA EC_{15} responses of α2β3γ2S receptors expressed in HEK293 cells

A. and B. Bar charts outlining the degree of potentiation achieved by co-application of vehicle (V), 100 nM THDOC (T), 500 nM diazepam (D) or both THDOC and diazepam (T + D) with 0.5 µM GABA (G). Responses are normalised to the preceding peak response to 0.5 µM GABA alone. Effects of THDOC and diazepam are additive in wild type receptors (A, n=11 cells); THDOC has no effect on α2^{Q241M} mutant receptors (B, n=6 cells). Significant effects of treatments are highlighted: ** $p < 0.01$ effect of substance vs. G alone; ++ $p < 0.01$ effect of T + D vs. response to T alone or D alone (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment).

C. Representative membrane currents for wild-type (blue) and α2^{Q241M} mutant (grey) α2β3γ2S receptors expressed in HEK293 cells.

3.2.3. Generating an $\alpha2^{Q241M}$ knock-in transgenic mouse

The process of generating the mouse strain is described more fully in *Section 2.2.1;* this stage of the project was performed by performed by Mike Lumb, a molecular biology technician in the lab, in collaboration with GenOway (Lyon, France). Briefly, wild-type exon 8 of α2 subunit genomic DNA in embryonic stem (ES) cells was precisely replaced with exon 8 containing the desired base-pair changes (CAA to ATG) for point mutation Q241M. ES cells that have successfully undergone homologous recombination were used to generate a transgenic mouse line with the $\alpha2^{Q241M}$ mutation on a C57BL/6J background.

Homozygous (hom) knock-in mice are viable and fertile, and there are no overt effects of the mutation on the general appearance of the mice. Animals were maintained as heterozygous (het) breeding pairs, to allow comparison of hom knock-ins with wild-type (wt) littermates. This is preferable over separately maintaining wt and hom lines, where genetic drift between the two populations could contribute to, or account for, any observed phenotypic differences (Bailey, 1982).

3.2.4. $\alpha2^{Q241M}$ has no effect on the expression of $GABA_A$ receptor subunits α1-α4 in Western blot assays

Total protein was isolated from four brain regions of interest (cortex, cerebellum, hippocampus and nucleus accumbens (NAcc)) as described in *Section 2.3.1*. Equal amounts of protein from six wt, six het and six hom males were subjected to Western blot analysis for each of the four $GABA_A$ subunits α1, α2, α3, and α4 (where detectable) using subunit-selective antisera (subunit specificity of antibodies was confirmed by blotting recombinant receptors – for details see *Section 2.3.2*).

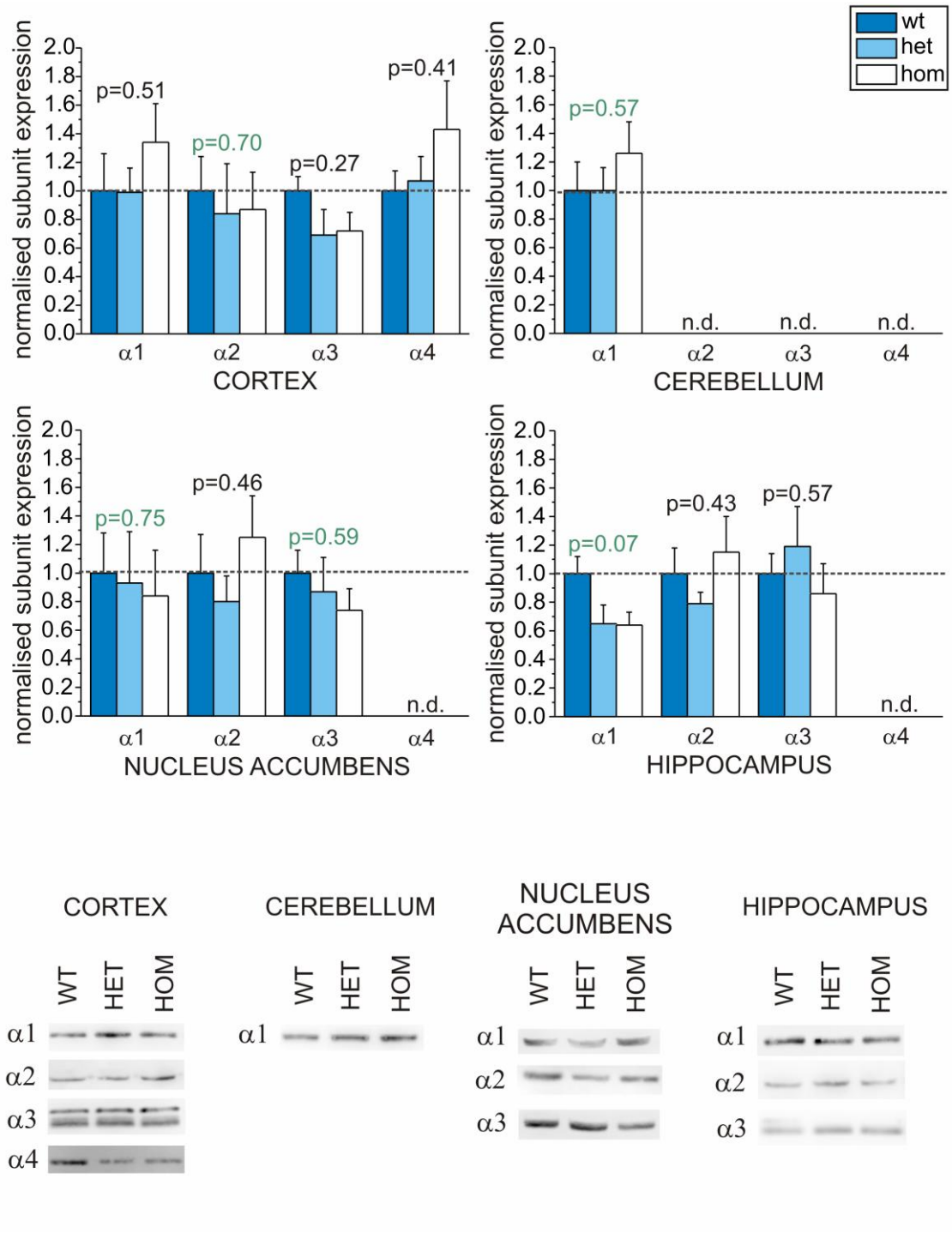

Figure 3.3 – No change in expression of $GABA_A$ subunits α1-α4

Bar charts detail the relative expression levels of each $GABA_A$ receptor subunit in protein samples from each brain area. There are no significant effects of genotype on expression of any subunit in any brain area tested (p values in black indicate results of parametric one-way ANOVA, those in green refer to non-parametric Kruskal-Wallis test results). n.d. - not determined because no reliable signal was obtained with calibration blots. *Bottom*: representative Western blots.

The blotting conditions were optimised by running a series of titrations to determine the amount of total protein which, when blotted under the conditions outlined in *Section 2.3.2*, produces a signal in the middle of the dynamic range of the detection system. When working under such conditions, one maximises the chances of detecting any up- or down-regulation of $GABA_A$ subunit expression. As can be seen from results of the Western blot assay (*Fig. 3.3*), there are no significant effects of genotype on subunit expression for any of the detectable subunits in any of the brain areas assayed.

3.2.5. $\alpha2^{Q241M}$ has no effect on the expression of $GABA_A$ receptor subunits α1-α5 in quantitative immunofluorescence assays

Three mice of each genotype (wt, het, hom) were sacrificed with a lethal overdose of sodium pentobarbital and brains were fixed by cardiac perfusion as described in *Section 2.4.1*. Thin (40 µm) coronal sections were obtained from frozen brain, and subjected to immunofluorescent staining and confocal z-stack image acquisition. Representative example images for the three areas studied – CA1 region of the hippocampus, the dentate gyrus (DG) and the NAcc – are shown in *Fig. 3.4*. The CA1 images encompass the cell body layer and apical dendrites, the DG images encompass the granule cell layer and molecular layer of the medial blade, and NAcc images include both core and shell regions.

The staining for α1 in both hippocampal regions is similar, showing a mixture of punctate and diffuse staining, with some cells showing much more intense staining of cell bodies and dendrites than others. Such a distribution of α1 has been seen by other investigators staining under similar conditions, using a different antiserum (Benke *et al.*, 1994; Mtchedlishvili *et al.*, 2003). Staining for α1 in the NAcc was rather different, showing a variable number of bright

punctae against a high background. This led to problems with quantification, as discussed in more detail below.

Immunostaining for α2 was similar and strong in all three areas examined. Punctate staining is seen throughout cell body and dendritic layers of both regions of the hippocampus. Similar staining for α2 in the DG has been previously published (Benke *et al.*, 1994). Uniformly-distributed punctate staining is also seen throughout the NAcc core and shell (as would be expected from the work of Pirker *et al.* (2000)).

As expected from immunohistochemical work by other investigators (Sperk *et al.*, 1997), immunofluorescent staining for α3 is much less intense than α1 and α2, showing fewer bright punctae against a very low background. The expression of α3 was rather heterogeneous throughout the NAcc: some clusters of cells showing very intense staining, whilst other areas have fluorescence at background levels. There were no obvious structural correlations that could explain this variation – neither a difference in core vs. shell regions of the NAcc, nor a clear anterior-posterior pattern of staining between coronal slices. The only common feature noted was a thin bright band of staining surrounding a dark region which often correlated with the very edge of the shell (asterisks on *Fig. 3.4 C*), but this was not true of every slice (c.f. wt, het and hom images in *Fig. 3.4 C*, all representing expression in a similar position of the NAcc). To ensure the mean fluorescence measure of expression accounts for this patchy distribution, images of α3 in the NAcc were acquired with a lower power lens (20x magnification vs. 63x in all other images) to encompass a larger field of view.

The α4 subunit shows a stronger expression in molecular layer than the granule cell layer of the DG, with an almost opposite distribution in CA1 (i.e. intense diffuse staining in the cell bodies and proximal regions of dendrites, and less intense staining in the dendritic region). It was also noted that there was an anterior-posterior variation in α4 expression, with more intense staining of

coronal sections corresponding to more anterior slices. This subunit also appears to be expressed well in the NAcc, as was expected from immunohistochemical work (Pirker *et al.*, 2000), and shows a mixture of diffuse and punctate staining uniformly throughout the structure.

The α5 isoform shows strong diffuse staining of cell bodies in all three regions studied. Staining in CA1 is very much like that for α4, except that no clear anterior-posterior variation was noted. In both CA1 and DG images, α5 can also be seen to have punctate staining uniformly throughout the dendritic region. This pattern for hippocampal α5 staining is most consistent with that seen by Prenosil *et al.* (2006). Similar strong expression of α5 in cell bodies is noted for staining in the NAcc, with no obvious difference between core and shell regions. Interestingly, Wisden *et al.* (1992) fail to detect α5 subunit expression in the NAcc at the level of mRNA. However, Pirker et al. (2000) observed α5 subunit expression in the NAcc using immunohistochemistry, which is described as weak and diffuse; unfortunately an image of this staining is not presented in this work, precluding a detailed comparison with our α5 staining pattern.

Figure 3.4 – Immunofluorescent localisation of $GABA_A$ subunits α1-5 in coronal sections of hippocampus and nucleus accumbens *(images on following pages)*

A. Representative images for immunofluorescent staining in the CA1 region of the hippocampus. Images were acquired with the 63x lens at 1x zoom, and encompass the cell body layer and the apical dendrites. Scale bar, 30 µm.

B. Representative images for immunofluorescent staining in the dentate gyrus. Images were acquired with the 63x lens at 1x zoom, and encompass the granule cell layer and molecular layer of the medial blade. Scale bar, 30 µm.

C. Representative images for immunofluorescent staining in the nucleus accumbens. Images for α1, α2, α4 and α5 were acquired with the 63x lens at 1x zoom; images for α3 were acquired with the 20x lens at 1x zoom. Scale bars, 30 µm. Red asterisks highlight examples of where strong α3 subunit immunoreactivity corresponds with the outer edge of the nucleus accumbens shell.

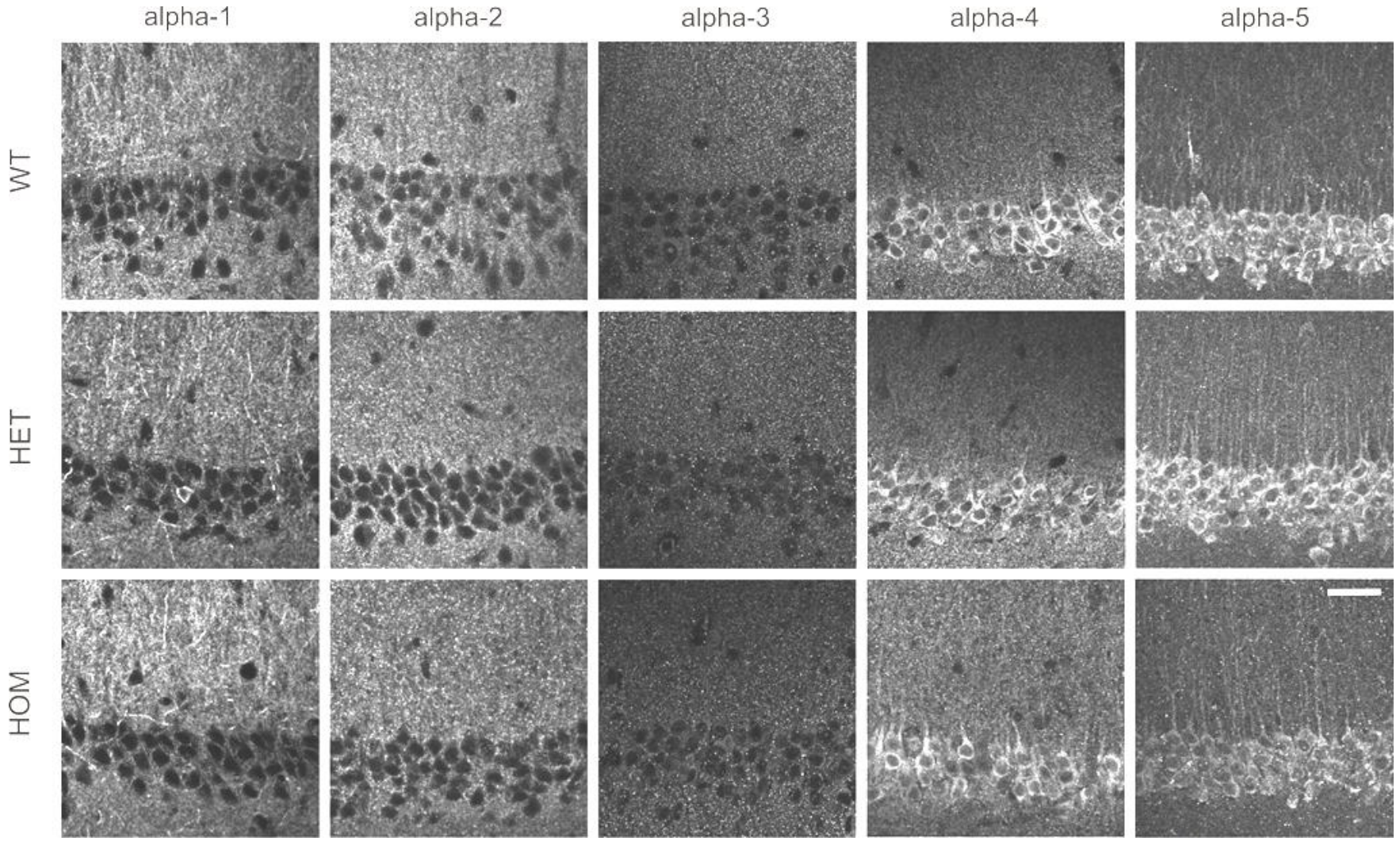

Fig 3.4 A – Hippocampal CA1 immunofluorescence: representative examples

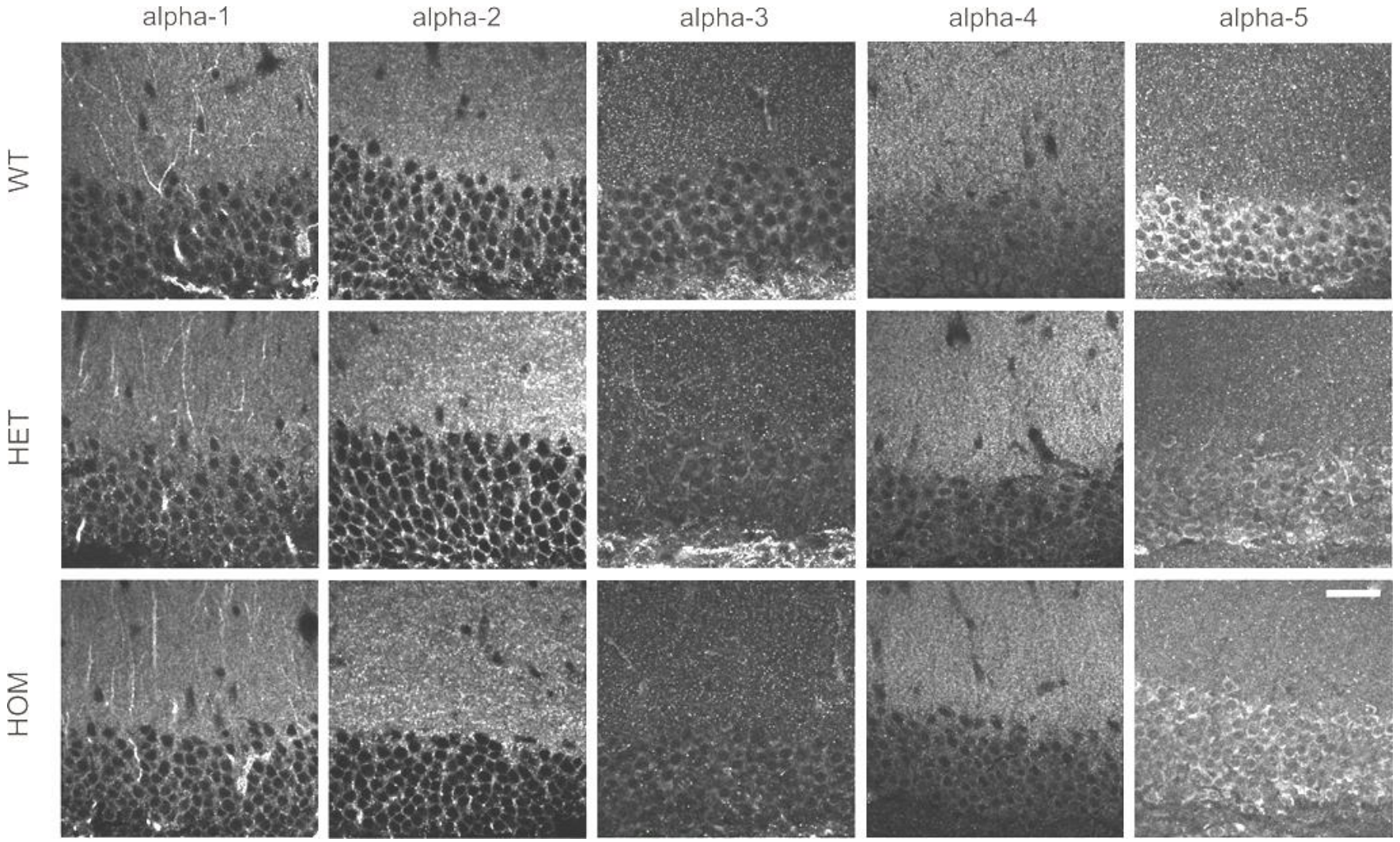

Fig 3.4 B – Dentate gyrus immunofluorescence: representative examples

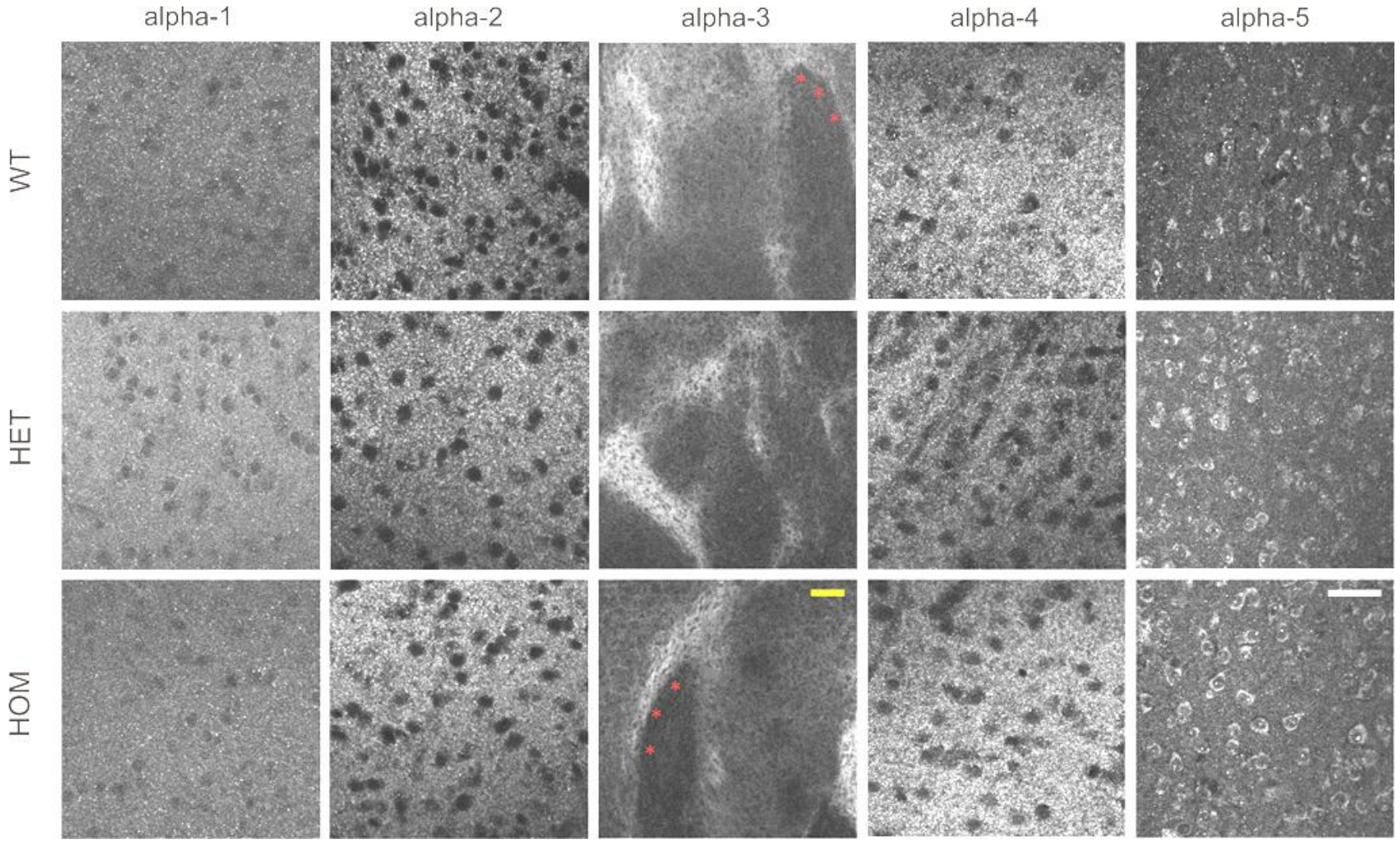

Fig 3.4 C – Nucleus accumbens immunofluorescence: representative examples

When quantifying the immunofluorescence, the simplest possible approach was employed to ensure that most of the image data was taken into account, and that analysis bias was minimised. For images of DG and CA1, z-stacks were split into two large regions of interest (ROIs) that encompass either the cell body layer or the dendritic region. The mean fluorescence intensity of the entire ROI was measured at three points in the z-stack (top, middle, bottom) and averaged. This process was performed for images from three coronal sections each from three animals of each genotype (i.e. mean values represent data from 9 z-stacks). This approach was chosen, rather than attempting to quantify expression in finer detail in individual cells, because there is no experimenter bias that would be involved in defining cells or dendrites for quantitation. To quantify expression in the NAcc, mean fluorescence intensity was measured for the entire image, rather than a specific ROI. The results of this simple method of quantitation are depicted in *Fig. 3.5*; no significant variation of subunit expression with genotype was detected for any of the subunits tested.

On closer inspection, one may note that some subunits show a trend towards a difference in subunit expression across genotypes: α3 expression tends to be reduced in the hom DG cell bodies and dendrites; α4 and α5 tend toward increased expression in hom DG dendrites; and α1 expression tends to be lower in the hom NAcc. These cases are therefore considered in more detail.

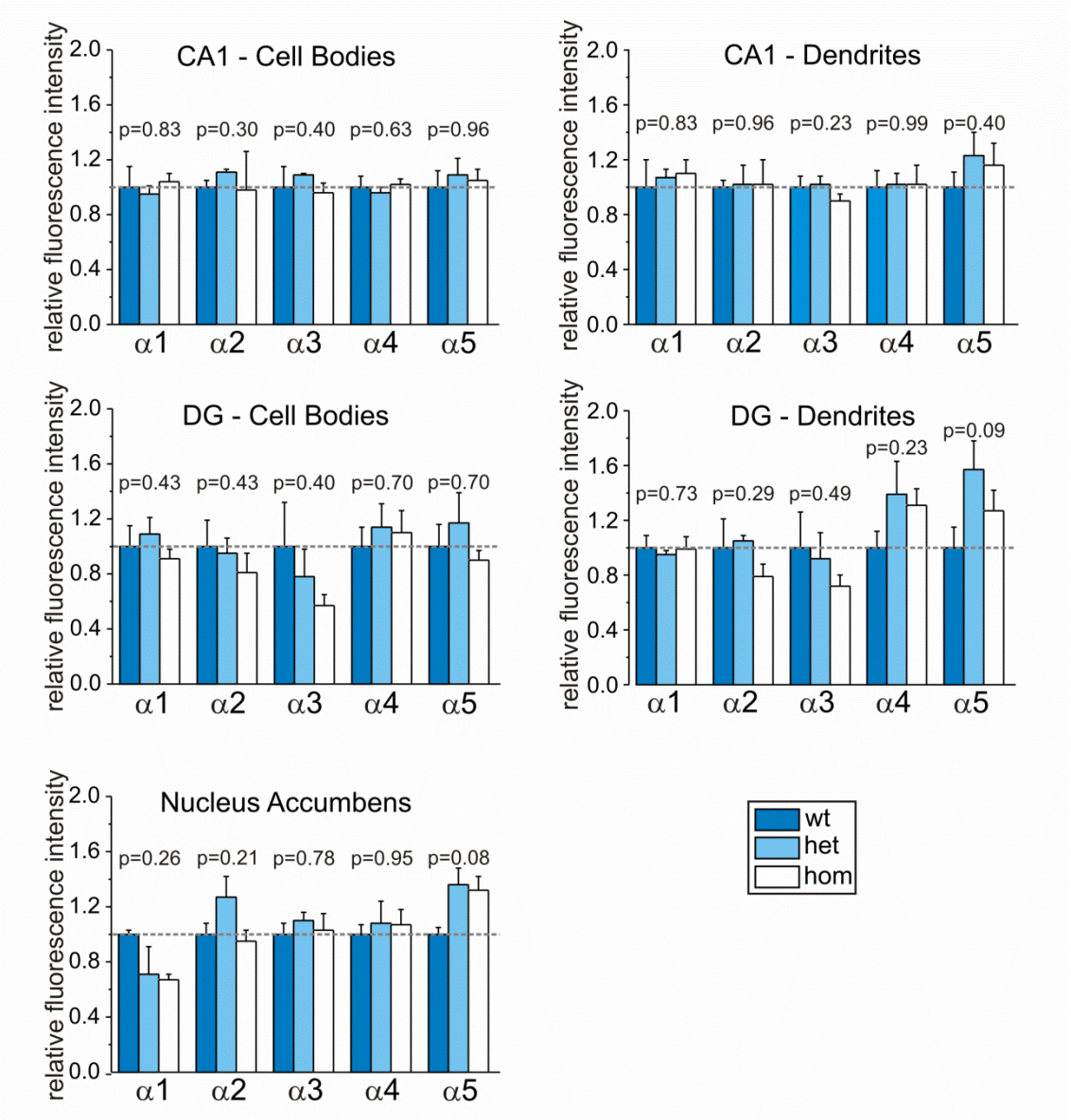

Figure 3.5 – Quantitation of immunofluorescent staining for $GABA_A$ subunits α1-α5 in coronal sections of hippocampus and nucleus accumbens

The relative mean fluorescence intensities for each subunit in each brain area are summarised in these bar charts. See *Section 2.4.3* for details. There are no significant effects of genotype on expression of any subunit in any brain area (p values refer to Kruskal-Wallis test results).

The contribution of background fluorescence to the mean fluorescence intensity values measured here can be a source of error. For example, there are few α1 punctae against high background fluorescence in the NAcc, so the background dominates the *mean fluorescence intensity*, and could account for the variation

seen in apparent expression. A similar problem is faced for α3 expression in the DG. In these cases, images were examined with the FociPicker3D plug-in in ImageJ, which identifies and quantifies immunopositive punctae by searching for local fluorescence maxima in three-dimensions. With these images, the plug-in is successful at identifying punctae against the background staining (see *Section 2.4.3* and *Fig. 2.3*). Results of this quantitation (*Fig. 3.6*) confirm that there are no changes in the density of immunopositive punctae (punctae per unit volume), the size of these punctae, or their intensity, across genotypes.

Interestingly, the number of α1-positive punctae was highly variable from slice-to-slice for the NAcc (e.g. one het animal gave slices with 112, 234 and 3874 punctae). There was no obvious positional correlation, either anterior vs. posterior or shell vs. core, that could explain this variability. Nevertheless, the average puncta densities across all images show no clear variation with each genotype, nor are those punctae any different in size or intensity between genotypes.

The FociPicker3D approach was not successfully applied to the α4 and α5 images because they contain a mixture of punctate and diffuse staining (see *Section 2.4.3* and *Fig. 2.3* for more details). Most α5 subunit staining is concentrated in the cell body layer in the DG, so the tendency toward fluorescence differences in the molecular layer is not a cause for concern. In contrast, α4 in the DG is more strongly expressed in the molecular layer, so a tendency toward increased mean fluorescence intensity in this region may represent an altered expression of this subunit in the knock-in mice. Statistically, however, this difference is not significant: the overall Kruskal-Wallis test produces a p value of 0.23. Individual pairwise comparisons also fail to reach significance (wt vs. het $p=0.20$, wt vs. hom $p=0.20$, het vs. hom $p>0.99$).

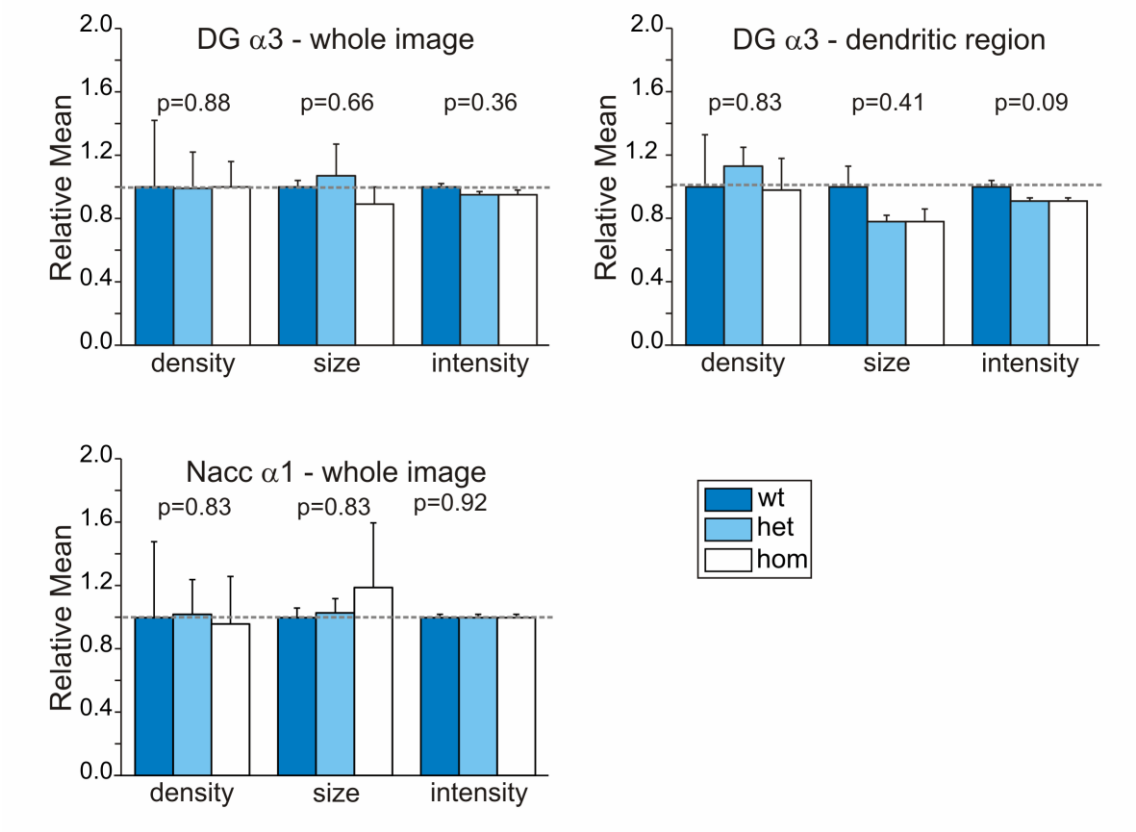

Figure 3.6 – Quantitation of immunofluorescent punctae for $GABA_A$ subunits α3 in the dentate gyrus and α1 in the nucleus accumbens

The relative mean density, size and intensities of immunopositive punctae for α3 and α1 subunits in the dentate gyrus (DG) and nucleus accumbens (NAcc), respectively, in coronal sections from wt, het and hom animals. Punctae were defined using the FociPicker3D plug-in for ImageJ software (see main text for details), using either entire z-stack images, or just the dendritic ROI (for DG α3 expression). There are no significant effects of genotype on expression of either subunit (p values refer to Kruskal-Wallis test results).

3.3. Discussion

3.3.1. $\alpha2^{Q241M}$ and $GABA_A$ receptor function

The mutation $\alpha2^{Q241M}$ has already been shown to abolish potentiation by the neurosteroid allopregnanolone (Hosie *et al.*, 2009). Here we extend this finding to the neurosteroid THDOC, which fails to potentiate EC_{15} GABA responses at 100 nM, when $\alpha2^{Q241M}$ is expressed in the receptor combination α2β3γ2S (*Fig. 3.2*). GABA sensitivity is retained in $\alpha2^{Q241M}$-mutant receptors, which also show similar maximal current responses (1 mM GABA responses: wt – 950 ± 100 pA, Q241M – 1400 ± 180 pA; p=0.07, unpaired t-test), which is consistent with unaltered cell surface expression of the mutant receptors in HEK293 cells. Assuming that these properties extend to $\alpha2^{Q241M}$ receptors when expressed *in vivo*, transgenic $\alpha2^{Q241M}$ knock-in mice would be preferred over α2 knock-out mouse to study the roles of the $GABA_A$ receptor α2 subunit in physiological and pharmacological responses to neurosteroids: in the knock-in, the retained expression and GABAergic function of the α2-type $GABA_A$ receptor would ensure that phenotypes of the mouse result only from the loss of neurosteroid function at that receptor.

Although not probed in this study, it is expected from previous work (Hosie *et al.*, 2006), that neurosteroids will retain action at the direct activation site. Nevertheless, the concentrations of neurosteroids required to achieve direct activation of the $GABA_A$ receptor are in the micromolar range – current measurements of neurosteroid levels suggest such concentrations will at best be at the very extremes of physiology (e.g. neurosteroid measures in pregnancy peak at 100 nM (Paul & Purdy, 1992)), and probably play little role in normal function of neurosteroids. Direct activation may become relevant during experiments probing responses to injected neurosteroid molecules, where levels at the synapse may well climb to those required for direct activation. In addition, the full effect of direct activation by neurosteroid appears to require concomitant binding and function of neurosteroids at the potentiation site (Hosie

et al., 2006). Both potentiation and direct activation by neurosteroids will therefore be defective at α2-type $GABA_A$ receptors in $α2^{Q241M}$ knock-in mice.

As was demonstrated for the equivalent mutation on α1-type $GABA_A$ receptors (Hosie *et al.*, 2006), $α2^{Q241M}$β3γ2S receptors have a wild-type-like response to diazepam potentiation (*Fig. 3.1*). This confirms that the mutation has not simply disrupted the ability of the GABA receptor to be potentiated *per se*, but that the lesion is specific to the action of neurosteroids via the potentiation site. The 1:1:1 ratio of cDNA expression vectors we used was sufficient in this study for expression and co-assembly of the γ2 subunit with α2 and β3, as indicated by their responses to diazepam (receptors lacking the γ2 subunit would be diazepam-insensitive (Pritchett *et al.*, 1989)).

3.3.2. No compensatory changes in $GABA_A$ receptor α subunit expression in $α2^{Q241M}$ mice

Whilst it is generally suggested that a knock-in mouse is preferable over a knock-out mouse, because there is a lower risk of compensatory up- or down-regulation of expression of various proteins, this should always be confirmed. For example, a microarray analysis of mice with an α1 or α2-subunit-directed knock-in mutation in two residues (S270H, L277A) – that disrupt ethanol sensitivity – reveal an array of changes to mRNA levels, implying multiple changes to many genes (Harris *et al.*, 2011). Furthermore, unlike the alcohol (Harris *et al.*, 2011) or benzodiazepine (Low *et al.*, 2000) site models, our transgenic model is losing sensitivity to a modulator that is present *endogenously*. Any compensatory changes in protein expression, particularly $GABA_A$ receptor subunits, resulting from knocking in the $α2^{Q241M}$ mutation would be interesting, but would also confound the investigation: do any of the phenotypes of the mouse result directly from loss of neurosteroid modulation of $GABA_A$ receptor α2 subunits, or indirectly as a consequence of compensatory alterations in the mouse?

There are several possible approaches in screening for compensatory changes. We chose to focus on methods that assay expression at the protein level (Western blot, immunofluorescence), rather than measuring mRNA levels (quantitative PCR, microarray) because it is changes of protein expression levels that are of functional importance. Our experiments have focussed solely on the expression levels of $GABA_A$ subunits α1-α5 as likely candidates for compensatory changes (the α6 subunit is not widely expressed, only being present in cerebellar granule cells and the cochlear nucleus (Laurie *et al.*, 1992a; Pirker *et al.*, 2000) and was not studied here). Analysing lysates of cortex, hippocampus, cerebellum and NAcc for α1-α4 expression showed no major global alterations in expression of these subunits. Not every subunit was detectable in all brain areas by Western blot (*Fig. 3.3*), and attempts to utilise an α5-selective antiserum in Western blots have proven unsuccessful, but this subunit was successfully assayed by immunofluorescence. Similarly, the anti-α4 antiserum was unable to produce a reproducible, quantifiable, signal for expression in whole-tissue lysates from hippocampus or nucleus accumbens, but this subunit is nicely detected by immunofluorescence. Subunits α1-α3 were successfully probed by both methods. Immunofluorescence allows subunit expression patterns to be examined in finer detail than Western blotting. Quantitative immunofluorescence focussed on subunits α1-α5 in the areas that were subjected to electrophysiological analysis (CA1, DG and NAcc, see *Chapter 4*). Overall, neither method has demonstrated any significant change in subunit expression for any of the five subunits.

When comparing immunofluorescent staining obtained in this study with immunostaining patterns in other published work, similarities and differences can be noted. In accord with work by Mtchedlishvili *et al.* (2003) and Benke *et al.* (1994), α1 antiserum stains some cells and processes in hippocampal slices much more brightly than others, but is otherwise characterised by widespread immunopositive punctae. The low abundance of α3 staining in the hippocampus is also in agreement with observations by others (Sperk *et al.*, 1997; Pirker *et al.*, 2000; Prenosil *et al.*, 2006). Other commonalities include the more intense

staining for α2 and α4 subunits in the molecular layer of the dentate gyrus (Benke *et al.*, 1994; Pirker *et al.*, 2000; Prenosil *et al.*, 2006). Poor expression for α1 subunits in the NAcc has also been demonstrated at mRNA (Sarviharju *et al.*, 2006) and protein (Pirker *et al.*, 2000) levels. In addition, the expression pattern for α2 in the DG resembles that in work published by others (Benke *et al.*, 1994).

Variable hippocampal expression patterns have been described for $GABA_A$ receptor α5 subunits in the literature. Our work and that of Prenosil *et al.* (2006) finds strong staining in cell bodies, whilst Sperk *et al.* (1997) and Pirker *et al.* (2000) demonstrate an absence of α5 from the cell body layers. Cell-body dominant staining for α5 subunits has also been observed in hippocampal sections from young (P7) rats (Ramos *et al.*, 2004), but these investigators then observe a pattern reminiscent of Sperk *et al.* (1997) and Pirker *et al.* (2000) in adult animals. Conversely, punctate staining of cell bodies have been observed in hippocampal slices from one month old mice (Farisello *et al.*, 2012). The staining patterns obtained in this study were highly reproducible and consistent from animal-to-animal and slice-to-slice. Perhaps this apparent variability between studies is a consequence of differences in animal age and species, and detection methods and antibodies used.

The expression of α subunit isoforms in the NAcc has been reported at the level of mRNA (Wisden *et al.*, 1992) and protein (Pirker *et al.*, 2000). The low level of staining we observe for α1 subunits is in agreement with these studies. Furthermore, our staining patterns for α2, α4 and α5 subunits in the NAcc are consistent with the description provided in the work by Pirker *et al.* (2000), who note that all three subunits show diffuse labelling, with the strongest signal for α2, and weakest for α5. The NAcc expression levels of α3 are described as low by both studies (Wisden *et al.*, 1992; Pirker *et al.*, 2000), but a figure is not available for a detailed comparison with our α3 staining pattern.

One important caveat of the approach used in this study is that they are reporting *total protein levels*, not just that at the cell surface. If immunofluorescence was performed without triton-x-100, only cell surface subunit expression would be revealed. However, the thickness of the slices used in this study (40 µm) demanded the use of triton-x-100 for antibody access to below-surface regions of the slice. Whilst immunostaining neuronal cultures may seem a suitable alternative approach to bypass the accessibility issue, the subunit expression patterns in a cultured neuron will be further removed from natural physiology than obtaining slices from fixed adult mouse brain. Another means to address this issue would be to co-stain slices for other markers; for example looking at the apposition of $GABA_A$ receptor punctae with a pre-synaptic marker, such as GAD65 or GAD67, would indicate which $GABA_A$ receptor punctae represent functional, synaptic $GABA_A$ receptors. Such an experiment would also help to demonstrate whether normal numbers of synaptic connections are formed in hom mice.

One must also remember that simply because there is no change in the *amount* of $GABA_A$ receptor α subunit, this does not guarantee that their modulation by other means is unaffected. Phosphorylation of $GABA_A$ receptors represents a major means of modulation *in vivo*, which can also interact with neurosteroid modulation (see *Sections 1.1.3* and *1.2.3*). Perhaps future experiments should probe $GABA_A$ receptor phosphorylation states in lysates from the $\alpha2^{Q241M}$ mice (e.g. using a phospho-specific antibody in Western blot).

Taken overall, the Western and immunofluorescence data presented here suggest that the phenotypes of this transgenic mouse are not a result of large changes in protein expression of the $GABA_A$ subunits α1-α5. It would be surprising if loss of neurosteroid function at α2-type $GABA_A$ receptors is not accommodated for in some way, and so it may be sensible to consider using a microarray or proteomic approach to screen for differences in expression patterns in hom vs. wt mice. Any ‘hits’ from this screen could then identify targets for further validation. Furthermore, we have not assessed the levels of

neurosteroid in the mouse strain, which may represent another avenue for compensatory changes.

3.3.3. Endogenous neurosteroids may modulate the in vivo *response to other $GABA_A$ receptor potentiators*

Retained sensitivity of the Q241M mutant receptors to potentiators such as benzodiazepines (*Fig. 3.1*) and pentobarbital (Hosie *et al.*, 2006) suggests that these compounds could be useful for positive controls in this study – for example if the anxiolytic response to neurosteroids is diminished in the $\alpha2^{Q241M}$ mice, is this a specific consequence of losing the neurosteroid potentiation site, or is there some general defect in the anti-anxiety circuitry? If the mice retain normal responses to diazepam or pentobarbital, then the latter hypothesis could be discounted.

However, there are a few complications to note. Firstly, neurosteroids are endogenous molecules, and so any modulator injected into a mouse will have its effects on $GABA_A$ receptors on top of the baseline modulation by endogenous neurosteroids. Secondly, certain benzodiazepines induce an increase in endogenous synthesis of neurosteroids, and these have been shown to underlie a component of the full *in vivo* response to these compounds (e.g. midazolam's anti-seizure activity is partly mediated by neurosteroid synthesis (Dhir & Rogawski, 2012)). By co-applying neurosteroid with diazepam in recombinantly expressed receptors (*Fig. 3.2*), it can be seen how the $\alpha2^{Q241M}$ mutation could affect responses to diazepam *in vivo*, where endogenous neurosteroids are present. This effect must therefore be considered in experiments in later chapters that use diazepam and pentobarbital as positive controls in these mice.

3.4. Conclusions

1. The $\alpha2^{Q241M}$ mutation specifically abolishes potentiation of heteropentameric αβγ $GABA_A$ receptor function by neurosteroids, whilst activation by GABA and potentiation by diazepam are unaffected when channels are studied in a heterologous expression system.

2. Transgenic knock-in mice have been generated, in which the wild-type copy of α2 genomic DNA has been precisely replaced with a Q241M point-mutated version of the receptor.

3. The $\alpha2^{Q241M}$ mutation has no overt effects on the expression of $GABA_A$ receptor subunits α1-α5 in the transgenic strain, whether present in the heterozygous or homozygous state – i.e. phenotypes of the knock-in mice can be attributed to the loss of neurosteroid potentiation (rather than altered $GABA_A$ receptor subunit expression and distribution).

4. Neurosteroids are present endogenously, and so the mutation may alter responses to other $GABA_A$ receptor potentiators in a whole animal.

Chapter 4: Electrophysiological consequences of losing neurosteroid modulation at $GABA_A$ receptor α2 subunits

4.1. Introduction

Neurosteroids can enhance tonic and phasic GABA transmission, with either response expected to reduce neuronal excitability by hyperpolarising the neuron and/or by shunting excitatory inputs. The roles of α2 subunits with regards to these responses can be defined using homozygous $\alpha2^{Q241M}$ mutant mice: any deficiency in the neurosteroid response could be attributed to the lack of neurosteroid potentiation at these receptors. To screen for any phenotypic effect of the $\alpha2^{Q241M}$ mutation, this investigation focussed on cells within the hippocampus and nucleus accumbens, which are known to strongly express the $GABA_A$ receptor α2 subunit (*Fig 3.4;* Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002; Mohler, 2006a).

4.1.1. GABAergic neurotransmission in the hippocampus

The hippocampus is implicated in many processes, including cognition, emotion, and the formation of spatial memories (Fanselow & Dong, 2010). Moreover, hippocampal pathologies are linked to epilepsy, anxiety disorders and depression (de Lanerolle *et al.*, 2003; Bannerman *et al.*, 2004; MacQueen & Frodl, 2011). The cytoarchitecture and circuitry of the hippocampus have been well defined and comprise two interlocking layers of pyramidal and granule cells (*Fig. 4.1*), both of which are under extensive GABAergic control. Hippocampal inputs into the hypothalamus are a major site for stress activation of the HPA axis (Brown *et al.*, 1999) and the hippocampus is proposed to be the site at which neurosteroids limit HPA axis activation by enhancing GABAergic inhibition in this area (see *Section 1.3.1*). Some of the anxiolytic function of

endogenous neurosteroids may involve their action in the hippocampus, and we propose that α2-type $GABA_A$ receptors will play a central role in this response. We have therefore examined inhibitory neurotransmission within the hippocampus of $α2^{Q241M}$ knock-in mice using whole-cell patch clamp electrophysiology. Experiments have focused on CA1 pyramidal cells (CA1 PCs) and dentate gyrus granule cells (DG GCs) within acute brain slices. Effects of the $α2^{Q241M}$ mutation are likely to be detected in these cell types, as both express the α2 subunit. Furthermore, the enrichment of α2 subunits at the axon initial segment of CA1 PCs places this receptor in an ideal position to inhibit action potential generation (Nusser *et al.*, 1996) and this subunit has already been demonstrated to play a significant role in generating IPSCs in these cells (Prenosil *et al.*, 2006). Neurosteroid-mediated potentiation of these receptors may therefore have profound consequences for firing of these neurons within the hippocampal circuit.

A number of observations suggest that tonic currents may dominate in mediating the anxiolytic response to endogenous neurosteroid. The increased neurosteroid efficacy at δ-containing receptors makes them better poised to respond to low levels of endogenous neurosteroids (Belelli *et al.*, 2002; Brown *et al.*, 2002; Stell *et al.*, 2003). Furthermore, the anxiolytic response to neurosteroid is impaired in δ-/- mice (Mihalek *et al.*, 1999), and Shen *et al.* (2007) find that reduced tonic transmission in the hippocampus induces anxiety during puberty in female mice. This may lead to the assumption that α2 subunits, which are thought to traffic to synaptic, as opposed to extrasynaptic, sites (Nusser *et al.*, 1996; Prenosil *et al.*, 2006), will play little role in neurosteroid-mediated anxiolysis. However, we are unaware of any evidence directly excluding α2 subunits from passing tonic currents. Indeed, synaptic receptors could be recruited to support tonic inhibition under certain conditions (e.g. increased ambient GABA and/or endogenous neurosteroid levels in the synapse could tonically open these channels). Furthermore, receptors can move into and out of synaptic sites by lateral mobility within the membrane (Thomas *et al.*, 2005); when present peri- or extra-synaptically, classically 'synaptic' α2-type receptors could be involved in passing tonic currents, if they

are activated by this exposure to ambient GABA. Indeed α5βnγ2 receptors have such a dual role, being responsible not only for CA1 PC tonic currents (see *Section 1.1.1*), but also for a proportion of IPSCs within these cells ('GABAslow' IPSCs (Banks *et al.*, 1998; Prenosil *et al.*, 2006)). Finally, roles for synaptic transmission in anxiety cannot be ruled out: synaptic α2-containing receptors mediate benzodiazepine anxiolysis (Low *et al.*, 2000), and several investigators find hippocampal IPSCs are modulated by physiologically-relevant concentrations of neurosteroid (e.g. Harney *et al.*, 2003). The contribution of α2-type $GABA_A$ receptors to both types of GABA current has therefore been assessed in our investigation.

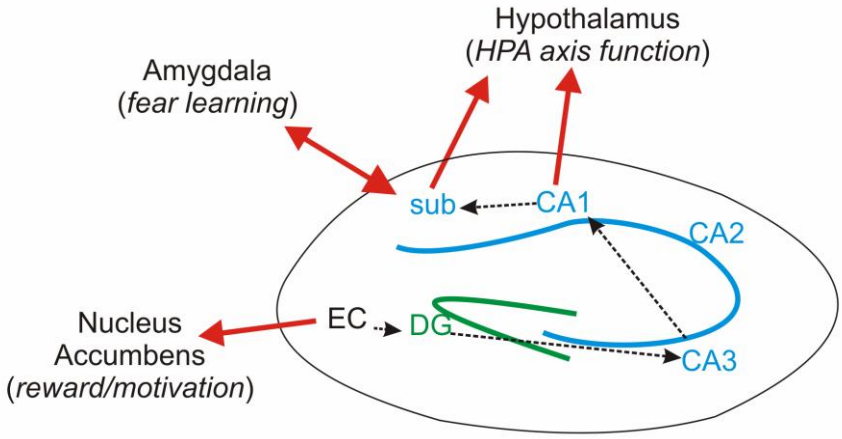

Figure 4.1 – Hippocampal networks relevant to anxiety and depression

The hippocampus has two major subdivisions, the *cornu ammonis* (CA) and the dentate gyrus (DG). Both components can be divided into 'cell body' and 'molecular' layers. Principal cells reside in the cell body layers: in the CA region, these are pyramidal cells (blue line) that can be divided into three regions (CA1-CA3), and in the DG, principal cells are granule cells (green line). Inhibitory GABAergic interneurons (not shown) are found in the molecular layers. Highlighted is the classical 'trisynaptic circuit' (black arrows), starting with inputs via the entorhinal cortex (EC), and ending at the subiculum (sub). Red arrows indicate some of the connections to other brain regions that are relevant to emotional state and mood disorders (Fanselow & Dong, 2010). Note that the connections are simplified, with many more reciprocal connections existing between the various subregions of the hippocampus.

4.1.2. GABAergic neurotransmission in the nucleus accumbens

The nucleus accumbens (NAcc) forms part of the mesolimbic dopamine pathway, which is believed to play roles in addiction and depression (see *Section 1.4.3*). NAcc activity is under extensive inhibitory control: the vast majority (95%) of neurons in the NAcc are GABAergic medium spiny neurons (MSNs), which not only project to outputs (such as the ventral palladium), but also make collateral connections with one another (Heimer *et al.*, 1997). Reduced neurosteroid and GABAergic function is implicated in the aetio-pathology of depression. We propose that the antidepressant functionality of neurosteroids could involve potentiation at α2-type $GABA_A$ receptors in the NAcc.

Synaptic currents in the NAcc have been shown to be, at least in part, mediated by α2-type $GABA_A$ receptors: Dixon *et al.* (2010) find mIPSCs are smaller and faster decaying in α2-/- mice, without any compensatory change in α subunit expression that could account for this change in IPSC properties. Furthermore, these investigators demonstrated that repeated stimulation of signalling through α2-type $GABA_A$ receptors (using Ro15-4513 in $\alpha2^{H101R}$ knock-in mice) induces behavioural sensitisation to Ro15-4513 and to cocaine, in a mechanism independent of dopamine release in the NAcc (Morris *et al.*, 2008; Dixon *et al.*, 2010). Thus, although signalling through α2-type $GABA_A$ receptors may not be directly involved in the rewarding effects of addictive drugs (Dixon *et al.*, 2010), synaptic signalling through this receptor does appear to be involved in strengthening behaviour toward the cues associated with drug taking (another aspect of addiction). By extension, it is possible that the same signalling is involved in learning to direct behaviour toward natural rewards, and so dysfunctional phasic transmission through α2-type $GABA_A$ receptors could contribute to depression. We therefore decided to characterise inhibitory neurotransmission in NAcc MSNs, using $\alpha2^{Q241M}$ mice to elucidate any roles for neurosteroid potentiation at α2 subunits in modulating both tonic and phasic transmission.

4.1.3. Modulation of synaptic and tonic GABAergic currents by benzodiazepines and neurosteroids

Spontaneous synaptic events (sIPSCs) and tonic currents have been assessed in CA1 PCs, DG GCs and NAcc MSNs within acute brain slices from our transgenic mouse strain. Properties of individual sIPSCs were assessed by fitting procedures to define the synaptic event rise time, amplitude, weighted decay time (τ_w) and charge transfer (area). The frequency of sIPSCs was also monitored by measuring the inter-event interval (I.E.I.). The level of tonic currents was measured by determining the change in root mean square (r.m.s.) current noise on application of 20 µM bicuculline. Baseline parameters were compared across genotypes to define any effects of the $\alpha2^{Q241M}$ mutation on basal inhibitory neurotransmission, both phasic and tonic.

Following stable baseline recording, $GABA_A$ receptor modulators THDOC or diazepam were applied. These modulators are expected to increase the duration of the decay phase of IPSCs: benzodiazepines (Otis & Mody, 1992; Bai *et al.*, 2001; Nusser & Mody, 2002; Dixon *et al.*, 2008), and neurosteroids and their analogues (Belelli & Herd, 2003; Harney *et al.*, 2003) have been shown to increase the decay times of miniature IPSCs (mIPSCs) from CA1 PCs, DG GCs and NAcc MSNs in acute brain slices. Reported effects on IPSC amplitude vary – sometimes an increase is observed (e.g. Prenosil *et al.*, 2006) – but often amplitude is unchanged (e.g. Otis & Mody, 1992); no increase in amplitude would indicate that synaptic receptors are saturated by the GABA released during baseline transmission. Any effects of these potentiators on IPSC frequency would indicate a pre-synaptic action, which has been observed in some, but not all neural circuits (e.g. allopregnanolone increased the frequency of GABAergic events in *Xenopus laevis* motorneurones (Reith & Sillar, 1997), but THDOC has no effect sIPSC frequency recorded from GCs in the cerebellum or DG (Stell *et al.*, 2003)). The sensitivity of tonic currents to neurosteroids and diazepam depend on the subunit composition of $GABA_A$

receptors passing the currents: responses to classical benzodiazepines require the presence of a γ subunit and absence of the benzodiazepine-insensitive α4 or α6 isoforms (Pritchett *et al.*, 1989; Wieland *et al.*, 1992); and although neurosteroids potentiate via the α subunit (i.e. will potentiate all $GABA_A$ receptor currents), the δ subunit confers a greater efficacy to neurosteroid potentiation than γ-containing equivalents (Belelli *et al.*, 2002; Brown *et al.*, 2002; Hosie *et al.*, 2009). Here we have assessed the effects of knocking-in $\alpha2^{Q241M}$ on the modulation of synaptic and tonic GABAergic currents by benzodiazepines and neurosteroids.

4.2. Results

4.2.1. $\alpha2^{Q241M}$ alters baseline inhibitory neurotransmission in acute slices of hippocampus and nucleus accumbens

All electrophysiological experiments started with a period of stable baseline recording, and data from these control epochs can be compared across genotypes – any genotypic differences will indicate baseline effects of the mutation. Because this mutation has no effect on GABA sensitivity (*Section 3.2.1*; Hosie *et al.*, 2006; Hosie *et al.*, 2009), nor an effect on $GABA_A$ receptor expression (subunits α1-α5, *Sections 3.2.4, 3.2.5*), one may predict that baseline inhibitory neurotransmission will be unaltered in knock-in mice compared to wt littermates. However, endogenous neurosteroids may play a significant role in modulating inhibitory neurotransmission recorded from brain slice tissue (Belelli & Herd, 2003; Puia *et al.*, 2003); if the α2 subunit is involved in responding to these endogenous steroids, genotypic differences in phasic or tonic GABA transmission will be expected at baseline. We have recorded from MSNs in the two main anatomical regions of the NAcc – 'shell' and 'core' – which are associated with distinct outputs, and functions (Heimer *et al.*, 1997; Shirayama & Chaki, 2006). However, we found no core vs. shell differences in baseline transmission (nor responses to THDOC or diazepam), so these data have been combined in the analyses below.

Phasic inhibition

Loss of neurosteroid potentiation at α2 subunits has some significant effects on baseline inhibitory synaptic neurotransmission measured in all three cell types (*Table 4.1*), most consistently a 20-30% decrease in τ_w decay time for recordings from homozygous animals compared to wild-types (*Fig. 4.2*). The $\alpha2^{Q241M}$ mutation also has a significant effect on τ_w when present in the heterozygous state for CA1 PCs, and tends toward an effect in het NAcc MSNs, although this difference is not quite significant (NAcc MSN, wt vs. het; p =

0.062, Behrens-Fisher test). Frequencies (measured by inter-event intervals) and amplitudes of sIPSCs are unaffected by genotype in recordings from any of the cell types. There is a slight tendency for sIPSC rise times to be faster in knock-in animals, although the only significant reductions are for hom vs. wt CA1 PCs and het vs. wt NAcc MSNs. The charge transfer per sIPSC (area values in *Table 4.1*) correlates well with τ_w, tending to be decreased in recordings from knock-ins vs. wild-types. Reductions in sIPSC area vs. wild-types are significant for het CA1 PCs and NAcc MSNs, and for hom DG GCs.

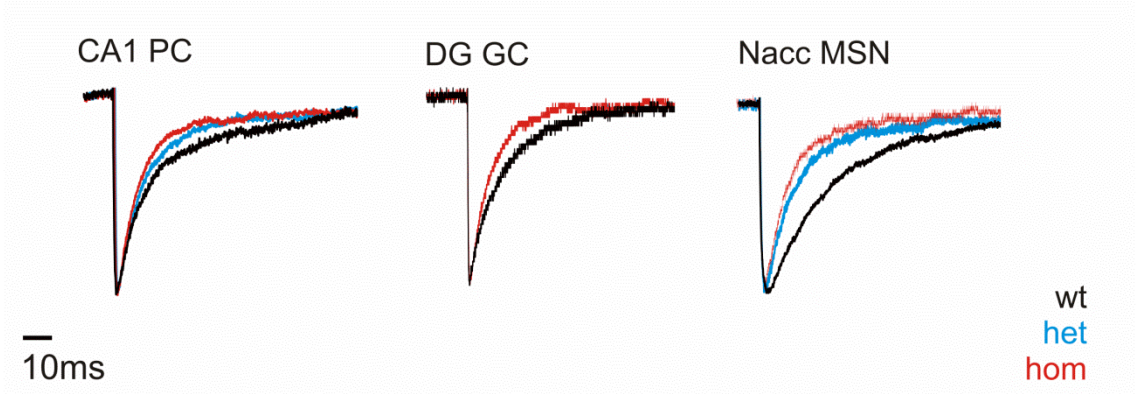

Figure 4.2 – α2^{Q241M} speeds the decay of baseline IPSCs

Representative baseline sIPSCs recorded from CA1 PCs, DG GCs and NAcc MSNs of each genotype (each event represents an average of at least 100 individual IPSCs). The events have been displayed with a peak-scaled amplitude to allow comparison of IPSC decays. Events in cells from wt animals (black) are slower to decay than those from het (blue) and hom (red) animals.

Baseline sIPSC Parameters		wt	het	hom
CA1 PCs (25 wt, 15 het, 15 hom)	**rise time (ms)**	1.85 ± 0.09	1.59 ± 0.09	1.49 ± 0.10*
	amplitude (pA)	-31.6 ± 1.4	-31.9 ± 1.5	-36.5 ± 1.9
	τ_w (ms)	15.2 ± 0.5	11.8 ± 0.4***	11.5 ± 0.6***
	area (pA.ms)	-383 ± 19	-304 ± 14**	-348 ± 26
	I.E.I. (ms)	202 ± 27	146 ± 37	145 ± 27
DG GCs (19 wt, 21 hom)	**rise time (ms)**	2.02 ± 0.13	-	1.84 ± 0.13
	amplitude (pA)	-39.9 ± 2.3	-	-37.6 ± 1.6
	τ_w (ms)	19.0 ± 1.0	-	13.7 ± 0.7****
	area (pA.ms)	-654 ± 69	-	-424 ± 26***
	I.E.I. (ms)	595 ± 134	-	401 ± 44
NAcc MSNs (32 wt, 45 het, 20 hom)	**rise time (ms)**	2.45 ± 0.09	2.13 ± 0.07*	2.43 ± 0.19
	amplitude (pA)	-46.8 ± 2.3	-42.9 ± 2.0	-45.8 ± 3.4
	τ_w (ms)	26.5 ± 1.6	22.8 ± 1.2	21.0 ± 1.7*
	area (pA.ms)	-1050 ± 74	-845 ± 70*	857 ± 101
	I.E.I. (ms)	478 ± 52	613 ± 64	744 ± 160

Table 4.1 – Comparing baseline sIPSCs recorded from acute brain slices

sIPSC properties (mean ± s.e.m.) from control epochs in the three cell types. In this table, and those that follow, numbers in brackets indicate number of cells (recordings in each case have been obtained from at least 4 animals). Statistically significant differences are highlighted: * - p<0.05, ** - p<0.01, *** - p<0.001, and **** - p<0.0001 vs. wt. (all pairwise Behrens-Fisher comparisons for CA1 PCs and NAcc MSNs, unpaired t-tests for DG GCs).

Tonic inhibition

The baseline tonic currents recorded from all three cell types showed no consistent variation with genotype (*Table 4.2*). NAcc MSNs tend toward reduced amplitude with the $\alpha2^{Q241M}$ mutation (i.e. tonic current wt>het>hom), but there is no overall effect of genotype on tonic current (one-way ANOVA for effect of genotype, p=0.256). Genotype also has no effect on the tonic current observed in CA1 PCs (one-way ANOVA for effect of genotype, p=0.341), or DG GCs (unpaired t-test wt vs. hom, p=0.883).

Baseline tonic currents	wt	het	hom
CA1 PCs (20 wt, 14 het, 13 hom)	0.73 ± 0.11	0.80 ± 0.16	0.81 ± 0.16
DG GCs (19 wt, 21 hom)	0.31 ± 0.06	-	0.33 ± 0.09
NAcc MSN (18 wt, 18 het,17 hom)	0.61 ± 0.08	0.49 ± 0.07	0.40 ± 0.07

Table 4.2 – Comparing baseline tonic currents in slice recordings

Tonic currents (mean ± s.e.m.) measured in CA1 PCs, DG GCs and NAcc MSNs. Numbers represent the root mean square (r.m.s.) current noise (pA) attributable to $GABA_A$ receptor currents (i.e. the difference between the r.m.s. noise in control epochs and that recorded after bicuculline treatment). Numbers in brackets indicate number of cells (recordings in each case were obtained from at least 4 animals).

4.2.2. $\alpha2^{Q241M}$ specifically diminishes neurosteroid potentiation of synaptic inhibitory neurotransmission

Following stable baseline recording, $GABA_A$ receptor modulators THDOC or diazepam were applied. Effects of these compounds on sIPSC parameters are expressed as a percentage change from the control condition within that cell. To confirm that any observed effects were due to $GABA_A$ receptor potentiation, rather than, for example, an effect of the DMSO vehicle or the prolonged recording period, 'mock' experiments were performed (see *Section 2.6.2*). If α2

subunits are significantly involved in the neurosteroid response of these currents, the response to THDOC will be reduced in slices from knock-in animals. Because the sensitivity to diazepam should be unaffected by the $α2^{Q241M}$ mutation (*Section 3.2.1*), diazepam responses serve as a positive control for $GABA_A$ receptor potentiation, and any deficit in response may indicate some compensatory change is present in the knock-in mouse.

THDOC treatment of CA1 PCs, as expected, increased sIPSC decay time (*Fig. 4.3* and *Table 4.3*). In cells from all three genotypes, 100 nM THDOC has significant effects compared to 'mock'-treated cells, but effects of the 50 nM application were only significant for CA1 PCs from wt and het animals (for hom animals, 50 nM THDOC vs. mock, p=0.693). The extent of τ_w prolongation also appears to depend on genotype: the 100 nM THDOC response of cells from hom mice is significantly diminished compared to those from wt mice. The response of cells from het animals to 100 nM THDOC is similar in magnitude to wt, but there is a higher variance on this response, and data are not significantly different to responses recorded in either wt or hom CA1 PCs.

The pattern of THDOC concentration-effects on sIPSC area mirrored the changes seen for τ_w (*Table 4.3*) – i.e. THDOC tends to increase the charge transfer per event, and the effect appears smaller in cells from hom animals than het or wt. However, there is a higher variance on these data, and only overall effects of drug reach significance in two-way ANOVA analyses (effect of drug, $p<0.001$, $F=9.97$, 2 degrees of freedom (d.f.); effect of genotype, $p=0.249$, $F=1.43$, 2 d.f.; interaction $p=0.605$, $F=0.69$, 4 d.f.). None of the individual pairwise comparisons between groups are statistically significant after correction for multiple comparisons. There were no significant changes in rise-times in response to THDOC, and no clear trend for a change in the sIPSC amplitude with application of THDOC. There were no significant changes in event frequencies in response to application of THDOC to CA1 PCs from animals of any genotype.

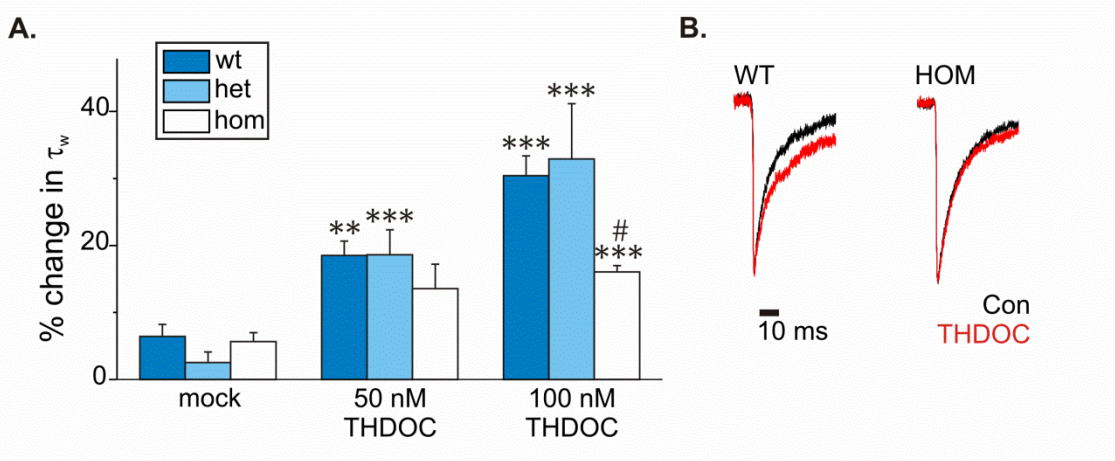

Figure 4.3 – THDOC effects on sIPSC decay time in CA1 pyramidal cells are diminished in recordings from homozygous knock-ins

A. Bar chart detailing concentration-response relationships for THDOC effects on τ_w decay time for sIPSCs recorded from CA1 pyramidal cells in acute hippocampal slices from animals of each genotype. ** p<0.01 and *** - p<0.001 for effect of THDOC vs. mock, # - p<0.05 for wt vs. hom (all pairwise Behrens-Fisher comparisons).

B. Representative sIPSCs for CA1 PCs from wild-types and homozygotes. The averaged control sIPSC (black) is superimposed on the averaged sIPSC after equilibration with 100 nM THDOC (red). Each event represents an average of at least 100 individual IPSCs, and is displayed with amplitude peak-scaled to allow comparison of IPSC decays.

CA1 PC sIPSCs: drug effects		wt	het	hom
rise time	**mock**	-2.7 ± 4.7 (n=7)	2.3 ± 5.3 (n=4)	-2.0 ± 4.1 (n=4)
	50 nM THDOC	8.3 ± 6.9 (n=6)	3.8 ± 4.5 (n=5)	3.2 ± 4.3 (n=6)
	100 nM THDOC	8.1 ± 4.1 (n=12)	9.8 ± 1.9 (n=6)	0.5 ± 3.1 (n=5)
amplitude	**mock**	-2.8 ± 4.3 (n=7)	-4.8 ± 8.7 (n=4)	-8.1 ± 5.8 (n=4)
	50 nM THDOC	18.3 ± 5.7 (n=6)	-1.3 ± 3.0 (n=5)	1.2 ± 2.8 (n=6)
	100 nM THDOC	6.3 ± 7.6 (n=12)	10.9 ± 7.5 (n=6)	4.8 ± 6.6 (n=5)
τ_w	**mock**	6.4 ± 1.8 (n=7)	2.5 ± 1.6 (n=4)	5.7 ± 1.4 (n=4)
	50 nM THDOC	18.5 ± 2.2 (n=6)**	18.6 ± 3.7 (n=5)***	13.6 ± 3.7 (n=6)
	100 nM THDOC	30.4 ± 3.0 (n=12)***	32.9 ± 8.2 (n=6)***	16.0 ± 1.0 (n=5)*** #
area	**mock**	4.1 ± 4.5 (n=7)	-0.8 ± 10.6 (n=4)	-2.9 ± 7.2 (n=4)
	50 nM THDOC	40.5 ± 9.0 (n=6)	19.1 ± 5.2 (n=5)	16.1 ± 3.7 (n=6)
	100 nM THDOC	37.2 ± 9.6 (n=12)	50.9 ± 18.6 (n=6)	22.8 ± 8.9 (n=5)
I.E.I	**mock**	13.3 ± 7.4 (n=7)	-20.1 ± 13.2 (n=4)	0.74 ± 27.9 (n=4)
	50 nM THDOC	80.0 ± 57.7 (n=6)	15.4 ± 8.0 (n=5)	8.2 ± 3.4 (n=6)
	100 nM THDOC	22.7 ± 6.5 (n=12)	28.2 ± 31.5 (n=6)	6.7 ± 5.9 (n=5)

Table 4.3 – THDOC-induced changes CA1 PC sIPSC parameters

Percentage changes (mean ± s.e.m.) in CA1 PC sIPSC parameters in response to THDOC application. ** p<0.01 and *** - p<0.001 for effect of THDOC vs. mock, # - p<0.05 for wt vs. hom (all pairwise Behrens-Fisher comparisons).

As expected, the $\alpha2^{Q241M}$ mutation diminished the sensitivity of synaptic receptors to the neurosteroid THDOC in recordings from CA1 PCs. The impact of genotype was apparent at the higher concentration of the neurosteroid, so experiments in DG GCs and NAcc MSNs focused only on 100 nM THDOC. These experiments also included diazepam as a positive control. The concentration of diazepam chosen, 500 nM, induces a similar level of potentiation as 100 nM THDOC when applied to recombinant $GABA_A$ receptors expressed in HEK293 cells (*Fig. 3.2*). In slices from wt animals, both substances have similar significant effects on sIPSC decay times in recordings from DG GCs and NAcc MSNs (*Fig. 4.4*, *Table 4.4*, *Table 4.5*). Consistent with our observations in CA1 PCs, the decay time prolongation by 100 nM THDOC is significantly reduced in DG GCs and NAcc MSNs from hom animals. In contrast, diazepam prolongation of sIPSC decay time is unaltered in DG GCs and NAcc MSNs from the homozygous knock-in animals compared to those of wt animals. These data are therefore consistent with the effects of the $\alpha2^{Q241M}$ mutation examined in HEK293 cells – disrupting neurosteroid potentiation without any effect on benzodiazepine sensitivity.

As with CA1 PCs, 100 nM THDOC and genotype have no significant effects on rise time, amplitude, area or frequency of sIPSCs recorded from DG GCs; there is also no significant effect of 500 nM diazepam on these parameters (*Table 4.4*). Two-way ANOVA analysis of the area data reveal a significant effect of treatment ($p=0.034$, $F=3.75$, 2 d.f.), but no overall effect of genotype ($p=0.307$, $F=1.08$, 1 d.f.), and no interaction effect ($p=0.207$, $F=1.65$, 2.d.f.). None of the individual pairwise comparisons survive correction for multiple comparisons, but some of the unadjusted least significant difference (LSD) comparisons are of note (wt mock vs. wt THDOC, $p=0.035$; wt mock vs. wt diazepam, $p=0.023$; and THDOC wt vs. hom, $p=0.056$). It therefore seems that there are at least some tendencies for the changes in charge transfer to correlate with changes seen with τ_w (i.e. THDOC being effective in wt/ineffective in hom, and diazepam being effective in both wt and hom).

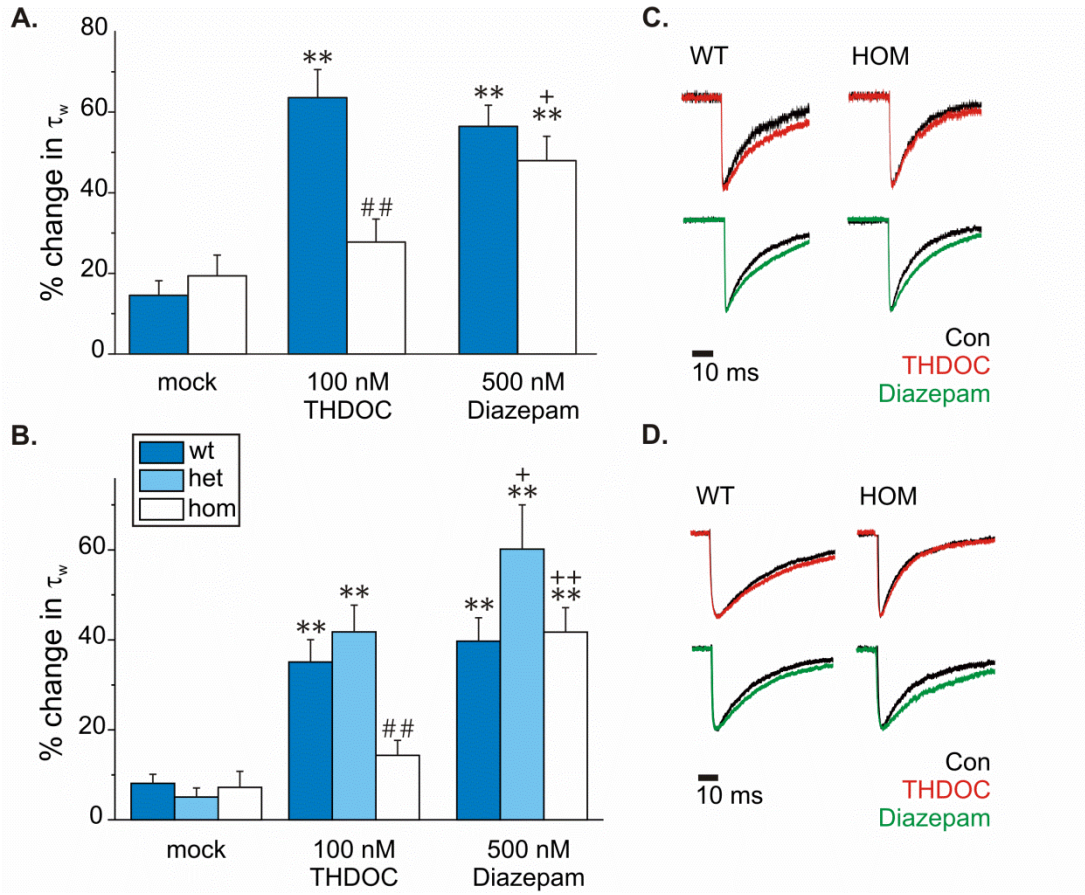

Figure 4.4 – Homozygous α2^{Q241M} specifically disrupts neurosteroid potentiation of sIPSCs in DG GCs and NAcc MSNs

A. THDOC and diazepam effects on τ_w decay time for sIPSCs recorded from DG GCs. ** - $p<0.01$ for effect of THDOC or diazepam vs. mock, + - $p<0.05$ for diazepam vs. THDOC, ## - $p<0.01$ for wt vs. hom (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment).

B. THDOC and diazepam effects on τ_w decay time for sIPSCs recorded from NAcc MSNs. ** $p<0.01$ for effect of THDOC or diazepam vs. mock treatment; + - $p<0.05$ and ++ - $p<0.01$ for effect of diazepam vs. THDOC; ## - $p<0.01$ for hom vs. het and hom vs. wt (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment).

C. and D. Representative sIPSCs for wild-type and homozygote DG GCs (C) and NAcc MSNs (D). The averaged sIPSC in the control epoch (black) is superimposed on the averaged sIPSC after equilibration with 100 nM THDOC (red) or 500 nM diazepam (green). Each event represents an average of at least 100 individual sIPSCs, and is displayed with amplitude peak-scaled to allow comparison of decays.

DG GC sIPSCs: drug effects		wt	hom
rise time	**Mock**	4.5 ± 1.8 (n=5)	13.3 ± 3.1 (n=7)
	100 nM THDOC	2.8 ± 7.9 (n=7)	7.4 ± 7.6 (n=7)
	500 nM diazepam	7.6 ± 4.5 (n=7)	21.7 ± 4.0 (n=7)
amplitude	**Mock**	17.3 ± 3.2 (n=5)	21.7 ± 8.8 (n=7)
	100 nM THDOC	25.2 ± 7.8 (n=7)	16.5 ± 3.9 (n=7)
	500 nM diazepam	30.0 ± 6.4 (n=7)	25.1 ± 7.1 (n=7)
τ_w	**Mock**	14.6 ± 3.6 (n=5)	19.4 ± 5.2 (n=7)
	100 nM THDOC	63.6 ± 7.0 (n=7)**	27.8 ± 5.7 (n=7) ##
	500 nM diazepam	56.5 ± 5.2 (n=7)**	47.4 ± 5.7 (n=7)** +
area	**Mock**	42.2 ± 8.0 (n=5)	60.2 ± 18.9 (n=7)
	100 nM THDOC	108.4 ± 21.2 (n=7)	53.8 ± 12.3 (n=7)
	500 nM diazepam	113.9 ± 15.1 (n=7)	105.4 ± 30.6 (n=7)
I.E.I	**Mock**	-14.2 ± 8.2 (n=5)	- 3.9 ± 11.1 (n=7)
	100 nM THDOC	19.0 ± 15.3 (n=7)	-7.4 ± 11.3 (n=7)
	500 nM diazepam	-16.3 ± 5.4 (n=7)	-12.0 ± 6.5 (n=7)

Table 4.4 – Changes in DG GC sIPSC parameters

Percentage changes (mean ± s.e.m.) in DG GC sIPSC parameters after application of THDOC or diazepam. ** - $p<0.01$ for effect of THDOC or diazepam vs. mock, + - $p<0.05$ for diazepam vs. THDOC, ## - $p<0.01$ for wt vs. hom (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment).

In recordings from NAcc MSNs, there are no effects of drug or genotype on the rise time, amplitude or frequency of sIPSCs (*Table 4.5*). Unlike the hippocampal recordings, however, the effects on charge transfer reach statistical significance: diazepam and THDOC significantly increase the areas of sIPSCs recorded from wt and het animals, whilst only diazepam is effective in cells from hom animals (i.e. no effect of THDOC in NAcc MSNs from hom mice).

NAcc MSN sIPSCs: drug effects		wt	het	hom
rise time	**mock**	9.3 ± 5.0 (n=12)	6.6 ± 3.9 (n=15)	7.4 ± 5.3 (n=7)
	100 nM THDOC	4.0 ± 4.2 (n=11)	5.6 ± 4.1 (n=21)	12.1 ± 11.0 (n=8)
	500 nM diazepam	11.7 ± 10.8 (n=9)	14.8 ± 8.8 (n=9)	14.5 ± 5.1 (n=5)
amplitude	**mock**	-2.9 ± 8.2 (n=12)	-6.1 ± 5.9 (n=15)	7.7 ± 3.8 (n=7)
	100 nM THDOC	-6.2 ± 13.0 (n=11)	3.4 ± 2.9 (n=21)	-2.3 ± 4.5 (n=8)
	500 nM diazepam	10.9 ± 6.1 (n=9)	7.5 ± 9.9 (n=9)	12.3 ± 9.7 (n=5)
τ_w	**mock**	8.1 ± 2.0 (n=12)	5.1 ± 2.0 (n=15)	7.2 ± 3.6 (n=7)
	100 nM THDOC	35.1 ± 4.9 (n=11)**	41.8 ± 5.9 (n=21)**	14.4 ± 3.3 (n=8)$^{\#\#}$
	500 nM diazepam	39.7 ± 5.2 (n=9)**	60.2 ± 9.8 (n=9)**$^{+}$	41.7 ± 5.5 (n=5)**$^{++}$
area	**mock**	4.9 ± 9.2 (n=12)	-2.9 ± 6.2 (n=15)	16.8 ± 8.3 (n=7)
	100 nM THDOC	29.9 ± 26.9 (n=11)*	46.0 ± 6.6 (n=21)***	14.7 ± 7.2 (n=8)
	500 nM diazepam	52.2 ± 6.1 (n=9)*	73.2 ± 17.6 (n=9)*	60.3 ± 11.8 (n=5)**$^{++}$
I.E.I	**mock**	24.5 ± 9.4 (n=12)	35.2 ± 20.9 (n=15)	13.6 ± 14.0 (n=7)
	100 nM THDOC	26.9 ± 12.1 (n=11)	16.7 ± 9.2 (n=21)	37.4 ± 34.7 (n=8)
	500 nM diazepam	4.3 ± 9.6 (n=9)	12.4 ± 11.5 (n=9)	-1.1 ± 11.5 (n=5)

Table 4.5 – Changes in NAcc MSN sIPSC parameters

Percentage changes (mean ± s.e.m.) in sIPSC parameters of NAcc MSNs after application of THDOC or diazepam. * p<0.05, ** p<0.01 and *** p<0.001 for effect of drug vs. mock treatment; + - p<0.05 and ++ - p<0.01 for effect of diazepam vs. THDOC; ## - p<0.01 for hom vs. het and hom vs. wt (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment (τ_w) or all pairwise Behrens-Fisher comparisons (area)).

4.2.3. $\alpha2^{Q241M}$ specifically diminishes neurosteroid potentiation of tonic inhibitory neurotransmission in dentate gyrus granule cells

DG GCs exhibited very little baseline tonic current in slices isolated from animals of either genotype (*Table 4.2*). Nevertheless, application of 100 nM THDOC significantly increased the r.m.s. current noise recorded from DG GCs of wt mice (*Fig. 4.5*, *Table 4.6*). This increase in noise is of $GABA_A$ receptor origin, because it is reversed by application of 20 µM bicuculline (*Fig. 4.5 B*). This effect of THDOC is not apparent for DG GCs in slices from hom animals, suggesting that it involves neurosteroid potentiation at α2-subunit-containing receptors. The lack of effect of 500 nM diazepam on r.m.s. noise (*Fig. 4.5 A*), further suggests that the receptors passing this tonic current are devoid of the γ subunit.

There were no significant variations of the r.m.s. current noise recorded from CA1 PCs (Kruskal Wallis test, p=0.138, 8 d.f) or NAcc MSNs (Kruskal Wallis test p=0.367, 8 d.f.) across any of the treatment groups (*Table 4.6*). There were no trends for any effect of THDOC or diazepam on r.m.s. noise recorded from 5-6 NAcc MSNs of any genotype, so analyses were not extended to the remaining 44 recordings.

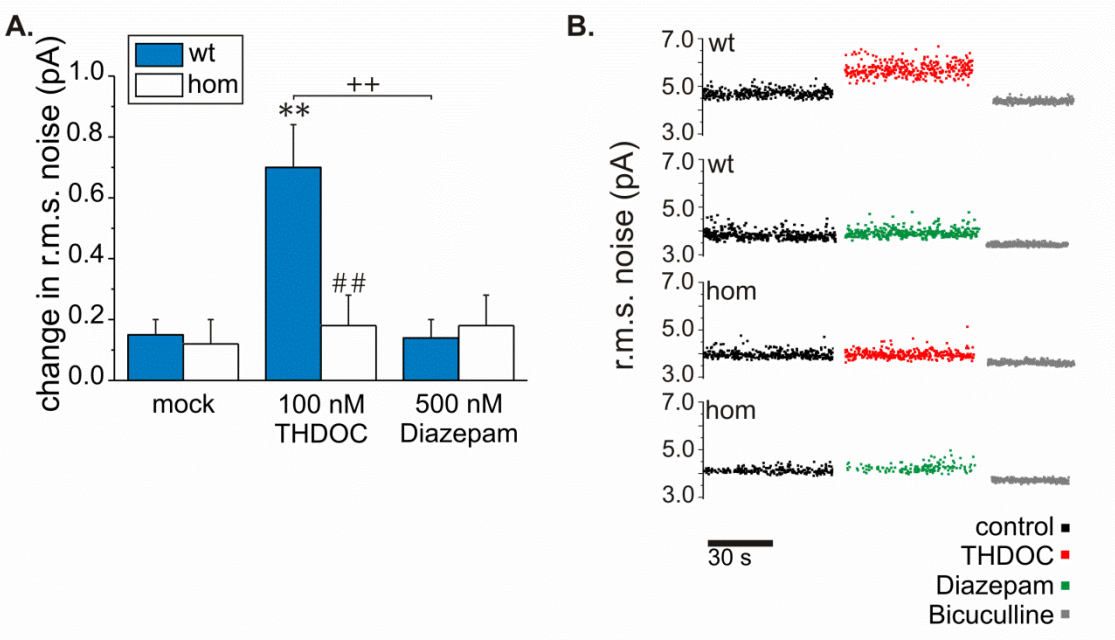

Figure 4.5 – Homozygous mutation α2^{Q241M} specifically disrupts neurosteroid potentiation of tonic currents in DG GCs

A. Bar chart detailing the change in r.m.s. current noise (mean ± s.e.m.) after equilibration of DG GCs with THDOC or diazepam. ** - p<0.01 for effect of THDOC vs. mock, ++ - p<0.01 for diazepam vs. THDOC, ## - p<0.01 for wt vs. hom (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment).

B. Representative examples of r.m.s. current noise after equilibration under the defined condition for wt and hom DG GCs.

Tonic currents: drug effects		wt	het	hom
CA1 PCs	**mock**	-0.23 ± 0.13 (n=6)	-0.18 ± 0.07 (n=4)	-0.05 ± 0.15 (n=3)
	50 nM THDOC	-0.16 ± 0.09 (n=4)	-0.14 ± 0.11 (n=5)	0.15 ± 0.17 (n=5)
	100 nM THDOC	0.10 ± 0.09 (n=10)	0.69 ± 0.37 (n=5)	0.31 ± 0.20 (n=5)
DG GCs	**mock**	0.15 ± 0.05 (n=5)	-	0.12 ± 0.08 (n=7)
	100 nM THDOC	0.70 ± 0.14 (n=7)**	-	0.18 ± 0.10 (n=7)$^{\#\#}$
	500 nM diazepam	0.14 ± 0.06 (n=7)$^{++}$	-	0.18 ± 0.10 (n=7)
NAcc MSNs	**mock**	-0.26 ± 0.10 (n=6)	-0.08 ± 0.03 (n=6)	0.13 ± 0.27 (n=6)
	100 nM THDOC	-0.06 ± 0.18 (n=6)	0.15 ± 0.12 (n=6)	-0.10 ± 0.11 (n=6)
	500 nM diazepam	-0.06 ± 0.07 (n=6)	-0.07 ± 0.14 (n=6)	0.08 ± 0.04 (n=5)

Table 4.6 – Comparing changes in tonic currents after equilibration with THDOC or diazepam

Summary of the changes (mean ± s.e.m.) in root mean square current noise (pA) after equilibration with drug in CA1 PCs, DG GCs or NAcc MSNs. ** - $p<0.01$ for effect of THDOC vs. mock, ++ - $p<0.01$ for diazepam vs. THDOC, ## - $p<0.01$ for wt vs. hom (ANOVA, multiple comparisons corrected with Benjamini-Hochberg adjustment).

4.3. Discussion

4.3.1. Effects of $\alpha2^{Q241M}$ on baseline synaptic and tonic GABA transmission

The $\alpha2^{Q241M}$ mutation has been shown to specifically ablate neurosteroid potentiation at α2β3γ2S receptors, without effect on responses to GABA (*Section 3.2.1*; Hosie *et al.*, 2006; Hosie *et al.*, 2009). Furthermore this mutation has been successfully introduced into our transgenic mouse strain without any obvious changes to the expression of $GABA_A$ receptor subunits α1-α5 (*Sections 3.2.4 and 3.2.5)*. One may therefore predict that the mutation would have no effect on baseline inhibitory neurotransmission within the transgenic mouse. In fact, results are to the contrary: baseline sIPSCs in knock-in mice decay faster than those of wt littermates, reducing the charge transfer per synaptic event. This does not necessarily indicate a compensatory alteration in response to the $\alpha2^{Q241M}$ mutation. Indeed the unchanged IPSC amplitude suggests that a similar number of $GABA_A$ receptors are expressed at the synaptic site, and/or that a similar amount of GABA is released per synaptic event. Unaltered sIPSC frequency would also suggest a lack of presynaptic alterations in the knock-in mice. We propose that the difference in baseline decay times is an indication for a role of endogenous neurosteroids in modulating inhibitory neurotransmission within the slice. There is already a precedent for such a role because inhibiting neurosteroid synthesis reduced the decay times of IPSCs recorded from pyramidal neurons of the neocortex (Puia *et al.*, 2003). Our results of faster decay times for sIPSCs from cells of hom $\alpha2^{Q241M}$ mice may therefore identify a role for α2-type $GABA_A$ receptors in responding to *endogenous* neurosteroids. If our model is correct, the difference between wt and hom IPSC decays should be ablated by removing endogenous neurosteroids from the slices.

Interestingly, some effects of the $\alpha2^{Q241M}$ mutation on baseline sIPSC parameters can also be seen in the heterozygous state (see *Section 4.2.1*). There are two α subunits per receptor (*Fig. 1.1*), and so two neurosteroid potentiation sites. Assuming that both copies of the subunit, $\alpha2^{M}$ and $\alpha2^{Q}$, are

expressed in equal amounts and co-assemble in an unbiased manner within cells of het animals, we would expect a binomial expression pattern of four combinations of wild-type and mutant copies of α2 subunits: $\alpha2^Q\alpha2^Q$, $\alpha2^Q\alpha2^M$, $\alpha2^M\alpha2^Q$ and $\alpha2^M\alpha2^M$. By comparing fits of one- and two-site models to experimental data, Hosie *et al.* (2009) suggested that one-site occupation is sufficient for full neurosteroid potentiation. Furthermore, using concatameric assemblies to control the $GABA_A$ receptor composition, Bracamontes and Steinbach (2009) showed that both α subunits are capable of contributing to neurosteroid potentiation. Effects of mutating each site individually were either small or insignificant, and potentiation by neurosteroid was only completely lost if both sites were mutated (Bracamontes & Steinbach, 2009). We would therefore expect het animals to respond 'normally' (like wt) to the endogenous neurosteroids in the slice (since only one in four receptor combinations is expected to be the insensitive double-mutant, $\alpha2^M\alpha2^M$). However, if there is a cell-to-cell variation in the relative expression levels of the neurosteroid-insensitive ($\alpha2^M\alpha2^M$) and sensitive ($\alpha2^Q\alpha2^Q$, $\alpha2^Q\alpha2^M$, $\alpha2^M\alpha2^Q$) receptor combinations in heterozygotes, cells with a higher proportion of $\alpha2^M\alpha2^M$ receptors would be expected to have a diminished response to endogenous neurosteroids within the slice. Our data on subunit expression cannot distinguish the wt and mutant alleles, but a quantitative PCR-based approach may help determine the relative expression levels of the two alleles in heterozygotes.

CA1 PCs, DG GCs and NAcc MSNs all showed very little response to bicuculline, suggesting that baseline tonic currents are small or absent from these cells under the conditions of our experiments. This is perhaps not a surprise: other investigators frequently require bath application of GABA, or a GABA reuptake inhibitor (such as NO-711), in order to achieve a consistent tonic current measurement in CA1 PC and DG GCs (e.g. Semyanov *et al.*, 2003). We have used neither manipulation, in order to preserve slices in a near-physiological state. Some investigators have observed tonic currents in these cells without altering extracellular GABA levels (e.g. Bai *et al.*, 2001; Nusser & Mody, 2002); it is difficult to say what condition may underlie the difference

between these investigations and ours. Perhaps there are some age- or sex-related variations in tonic currents; for example the expression of δ-subunit containing receptors, which dominate in DG GC tonic currents (see *Section 1.1.1*), increases with increases with age (Laurie *et al.*, 1992b). Our investigation focussed on young (P18-30) males, and we cannot discount the presence of more robust tonic currents at older ages, or in females, in these cell types within our mouse strain. In our investigation, the $\alpha2^{Q241M}$ mutation has no effect on the magnitude of the baseline tonic current (*Table 4.2*). This would imply that, at least under the conditions of our experiment, there is no involvement of $GABA_A$ receptor α2 subunits (or neurosteroid potentiation at these subunits) in generating baseline tonic currents. Furthermore, unchanged tonic currents would be consistent with a lack of compensatory changes in expression of extrasynaptic $GABA_A$ receptors in the knock-in mice.

4.3.2. $\alpha2^{Q241M}$ specifically diminishes neurosteroid potentiation of synaptic transmission

The clearest effect of THDOC and diazepam on the sIPSCs is a prolongation of decay times, which is seen in recordings from all three cell types. The lack of effect on IPSC amplitude suggests that GABA release during phasic transmission is already saturating postsynaptic receptors, and is consistent with observations of others (e.g. Otis & Mody, 1992). Effects on sIPSC area are consistent with those on decay time, but these effects are less robust, possibly because area depends on a combination of decay time (increases in all cells) and amplitude (although the average suggests no change, within individual cells, amplitude can increase, decrease or remain unchanged). A lack of effect of THDOC on inter-event interval is consistent with a lack of presynaptic effects of THDOC in the hippocampus and NAcc.

The strong effect we observe for 100 nM THDOC on τ_w from wt DG GCs may appear at odds with reports elsewhere of insensitivity of these cells to THDOC

(Stell *et al.*, 2003) and their low sensitivity to allopregnanolone (Belelli & Herd, 2003; Harney *et al.*, 2003). However, the neurosteroid sensitivity of IPSC decay time varies with age, receptor subunit composition, cellular differences in metabolism and relative kinase/phosphatase activities (see *Section 1.2.3*). Any one of these factors may account for the discrepancies between these observations. For example, DG GC IPSC sensitivity to neurosteroid declines with age in rats (Cooper *et al.*, 1999; Mtchedlishvili *et al.*, 2003), being highest during the first two postnatal weeks; responses to 'physiological' levels of THDOC (50 nM and 100 nM) are lost soon after (P17-21 (Cooper *et al.*, 1999)). Perhaps neurosteroid sensitivity declines at around P30 in mice, which would explain why IPSCs are responsive in our work (P18-30 mice) but not in that of Stell *et al.* (2003) (P30-P181 mice).

Since the $\alpha2^{Q241M}$ mutation ablates neurosteroid potentiation of α2β3γ2S receptor function in HEK293 cells, one would predict potentiation of GABAergic sIPSCs to also be defective in $\alpha2^{Q241M}$ knock-in mice. Focussing on the sIPSC τ_w response to THDOC, data are consistent with this prediction: sIPSC decay prolongation by 100 nM THDOC is diminished in hom knock-ins all three cell types examined. In NAcc MSNs and DG GCs, the response is ablated (i.e. no different to the responses of 'mock' treated cells); in CA1 PCs, neurosteroid sensitivity is significantly diminished, but not completely lost. The most likely explanation for residual response in CA1 PCs is the retained neurosteroid-potentiation functionality of other α subunit isoforms in these cells (α1, α3, α4 and α5 (*Fig 3.4A*; Wisden *et al.*, 1992; Prenosil *et al.*, 2006)). It would therefore seem likely that α2-type $GABA_A$ receptors are responsible for approximately 50% of sIPSCs recorded from CA1 PCs (decay time prolongation by 100 nM THDOC: 16% in hom vs. 30% in wt), and for the majority of sIPSCs in DG GCs and NAcc MSNs. Whilst the differential response in cells from hom mice could alternatively represent a compensatory change in response to the $\alpha2^{Q241M}$ mutation, subunit expression data would argue against this notion (*Section 3.3.2*). Furthermore, the IPSC decay time prolongation by 500 nM diazepam is undiminished by the $\alpha2^{Q241M}$ mutation in DG GCs and NAcc MSNs (*Fig. 4.4*). These data are consistent with unaltered function of synaptic $GABA_A$ receptors

(i.e. support a lack of compensatory changes). We therefore propose a key role for α2-type $GABA_A$ receptors in overall neurosteroid response of sIPSCs.

Neurosteroid and diazepam modulation of sIPSCs recorded from cells of het animals are generally the same as those observed in cells from wt mice. The response of het NAcc MSNs to 100 nM THDOC, assessed by τ_w prolongation or by increased event area, is no different to that seen in wt littermates. Similarly, het NAcc MSNs show a wt-like response to 500 nM diazepam. The response of het CA1 PCs to 100 nM THDOC are the same as for wt (although pairwise comparisons find no significant difference for het vs. hom and for het vs. wt). These data appear to be at odds with the effects of het $\alpha2^{Q241M}$ on baseline IPSC characteristics discussed in *Section 4.3.1*; the reason for this discrepancy remains unclear.

Interestingly, in NAcc MSNs from heterozygotes, the response to 500 nM diazepam is significantly higher than that for 100 nM THDOC. It is not clear why this is the case, because these doses of modulator achieve the same degree of potentiation of wt α2-type $GABA_A$ receptors expressed in HEK293 cells (*Fig. 3.2 A*). However, the diazepam and THDOC sensitivity of heterozygous $\alpha2^{Q241M}$ mutant receptors has not been examined in HEK293 cells; perhaps the concatamer-based approach of Bracamontes and Steinbach (2009) should be utilised to examine the effects of mutating a specific single neurosteroid potentiation site within the pentamer on diazepam sensitivity. Slice electrophysiology could also be extended to determine if this effect is specific to NAcc MSNs: the diazepam sensitivity of CA1 PCs of all three genotypes should be examined, whilst heterozygous animals should be added to the work in DG GCs.

4.3.3. $\alpha2^{Q241M}$ reveals a role for α2-type $GABA_A$ receptors in tonic currents

Application of 100 nM THDOC significantly increased the r.m.s. current noise recorded from DG GCs of wt mice (*Fig. 4.5*, *Table 4.6*). This increase in noise is of $GABA_A$ receptor origin, because it is reversed by application of 20 µM bicuculline (*Fig. 4.5 B*). This effect of THDOC is not apparent in DG GCs in slices from hom animals, suggesting that it involves neurosteroid potentiation at α2-type receptors. The lack of effect of 500 nM diazepam on r.m.s. noise (*Fig. 4.5 A*), further suggests that the receptors passing this tonic current are devoid of the γ subunit (Pritchett *et al.*, 1989). Potential receptor combinations accounting for this tonic current could therefore be α2βn and α2βnδ.

There were no significant effects of either THDOC or diazepam on the r.m.s. current noise recorded from CA1 PCs or NAcc MSNs. Under the conditions of our experiments, therefore, it seems that tonic GABA currents in both cell types are carried by receptors lacking the γ subunit (which would bestow diazepam insensitivity). It has been suggested that neurosteroid insensitivity of tonic currents indicates that receptors lack the δ subunit (Stell *et al.*, 2003), but 100 nM THDOC clearly has an effect on the synaptic (probably γ-type) receptors in the same cells, so even a γ-subunit-mediated tonic current would be expected to respond to THDOC. Perhaps the neurosteroid insensitivity of the extrasynaptic receptors in these cell types can be attributed to their phosphorylation state under the conditions of our experiment.

4.3.4. Limitations of the electrophysiological characterisation

Any experimental approach to measuring neurotransmission will itself perturb normal function, and the further removed the approach is from the intact brain, the greater the potential for departure from normality. Work in this thesis utilises brain slice tissue, which represents a state closer to *in situ* physiology than

using dissociated neurons in culture, because cells are maintained in their native local milieu, and many of their original synaptic inputs are retained. The properties of synaptic and tonic currents recorded form cells in brain slices can be influenced by the recording temperature, holding voltage, Cl^- loading and the addition of compounds that influence the ambient GABA concentration (Otis & Mody, 1992; Cooper *et al.*, 1999; Overstreet *et al.*, 2000; Semyanov *et al.*, 2003; Semyanov *et al.*, 2004; Houston *et al.*, 2009a). Importantly, manipulating extracellular GABA levels can determine which subunits contribute to that current, with a more prominent role for α5-type receptors in CA1 PCs at elevated GABA concentrations (Scimemi *et al.*, 2005; Prenosil *et al.*, 2006). It is therefore possible that the roles we propose for α2-type $GABA_A$ receptors only hold under the particular experimental conditions used. It would be interesting to expand our study of tonic current to conditions that will raise ambient GABA levels, to see if the neurosteroid-stimulated α2-dependent tonic current is retained. It would also be interesting to extend the investigation, to screen for the effects of $α2^{Q241M}$ within different cell types and at different developmental stages, particularly where GABAergic currents may be excitatory (Ben-Ari, 2002; Szabadics *et al.*, 2006; Chiang *et al.*, 2012).

Most experiments have utilised 100 nM THDOC to potentiate GABAergic transmission within the slice. This concentration represents the higher end of that which has been measured physiologically (Paul & Purdy, 1992; Concas *et al.*, 1998), and some investigators suggest it is better to focus on lower concentrations (less than 50 nM) to represent those seen more commonly during physiology (Harney *et al.*, 2003; Stell *et al.*, 2003). Interestingly, in our work, the 50 nM response of hom CA1 PCs does not seem to be lower than that in recordings from het and wt CA1 PCs (*Fig. 4.3*), which may suggest that the α2 subunit is only important in responses to higher neurosteroid levels. However, it is difficult to know whether the whole-tissue estimates of neurosteroid levels directly correlate with those found at the level of individual cells. Endogenous neurosteroids act in a paracrine manner, and applied neurosteroids can accumulate within cells (Li *et al.*, 2007), such that the concentration of compound in the cell surface membrane (i.e. at the level of the

$GABA_A$ receptor) may be in excess of that applied in the bath (Chisari *et al.*, 2010). Furthermore, Belelli and Herd (2003) find that local degradation to inactive metabolites may shape the response of different cells within hippocampal slices to the same concentration of bath-applied neurosteroid. Whether our observed neurosteroid-response deficit relates to physiological levels of these compounds therefore awaits methods to accurately measure their concentrations at synapses. Encouragingly, however, there are clear baseline effects of $\alpha2^{Q241M}$ on IPSC decay time. We propose that this identifies an endogenous neurosteroid tone within CA1 PCs, DG GCs and NAcc MSNs, that influences synaptic neurotransmission by function at α2-type $GABA_A$ receptors.

4.4. Conclusions

1. Baseline sIPSCs are faster-decaying in several cell types from hom mice vs. wt littermates, suggesting a role for endogenous neurosteroids in setting the duration of inhibitory synaptic transmission. By extension, hom mice are predicted to have a diminished inhibitory tone *in vivo*.

2. IPSC responses to diazepam are unperturbed by the $\alpha2^{Q241M}$ mutation in brain slices from the knock-in mice, consistent with a lack of compensatory alterations in the mouse strain.

3. Functional potentiation sites on $GABA_A$ receptor α2 subunits are required for a full response of synaptic events to neurosteroids in CA1 PCs, DG GCs and NAcc MSNs.

4. In DG GCs, $GABA_A$ receptor α2 subunits appear not to be involved in baseline tonic currents, but may be recruited to pass such currents at high THDOC concentrations. The receptors involved are likely to be peri- or extra-synaptic γ-subunit-lacking receptor combinations (α2βn and α2βnδ).

Chapter 5: Screening anxiety and depression phenotypes of α2^{Q241M} knock-ins

5.1. Introduction

5.1.1. Behaviour of α2^{Q241M} mice defines the physiological and pharmacological roles of neurosteroids acting at GABA$_A$ receptor α2 subunits

The α2^{Q241M} mice generated in this study provide a unique opportunity to elucidate the roles of α2-type GABA$_A$ receptors in mediating responses to both endogenous and injected neurosteroids. Levels of endogenous neurosteroids fluctuate during a number of normal and pathological settings, being raised by stress (Purdy *et al.*, 1991; Barbaccia *et al.*, 1996) and diminished in depression (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000), for example. Differences in baseline behavioural phenotypes of the α2^{Q241M} knock-ins, compared to wild-type littermates, are likely to be a consequence of losing regulation by these endogenous neurosteroids at α2-type GABA$_A$ receptors, especially given that we have not found any compensatory changes in expression of GABA$_A$ receptors α1-α5 that could account for changes in behaviour (*Chapter 3*).

Injected neurosteroids have a number of effects, including anxiolysis (Crawley *et al.*, 1986; Wieland *et al.*, 1991), antidepression (Khisti *et al.*, 2000), analgesia (Winter *et al.*, 2003), and sedation (Mendelson *et al.*, 1987), all of which imply that neurosteroids could prove useful therapeutics for a number of nervous system disorders. It remains to be determined whether, as for benzodiazepines, different GABA$_A$ receptor α-subunit isoforms are responsible for each specific behavioural effect of neurosteroids. Examining responses to injected neurosteroids in α2^{Q241M} mutant mice will demonstrate which behavioural effects listed above depend on potentiation at the GABA$_A$ receptor α2 subunit.

Behavioural studies in this thesis have focussed on paradigms that assess anxiety-like and depression-like behaviours within the α2^{Q241M} knock-in mouse strain.

5.1.2. $GABA_A$ receptor α2 subunits and neurosteroids in anxiety

$GABA_A$ receptor α2 subunits are key components of the anxiety circuitry in the CNS (see *Section 1.3.2*). Fundamental observations are that α2 expression is strong in brain areas linked to mood and anxiety, including cortex, hippocampus and amygdala (Wisden *et al.*, 1992; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002; Mohler, 2006a), and that knock-out of this subunit causes an anxiety phenotype (Dixon *et al.*, 2008). Furthermore, the anxiolytic response to benzodiazepines is thought to be mediated by α2-type $GABA_A$ receptors (Low *et al.*, 2000). If $GABA_A$ receptor α2 subunits are also central to the anxiolytic response to endogenous neurosteroids, one would predict a baseline anxiety phenotype for hom α2^{Q241M} mutants, which will be incapable of responding to these compounds at α2 subunits. Moreover, this defect would be predicted to impair anxiolytic responses to injected neurosteroids. These predictions were tested using two behavioural screens for anxiety-like behaviour: the elevated plus maze and light-dark box tests.

The elevated plus maze has been validated over many years as a screen for anxiety-like behaviour in mice (Lister, 1987; Hogg, 1996). This test, described in *Section 2.7.2*, exploits the aversive properties of open spaces. Mice are faced with a choice between exploring regions open to the environment and 'safer' enclosed regions, which supply thigmotactic cues. Drugs known to be anxiolytic in humans, including benzodiazepines, increase the number of entries into and percentage time spent on the aversive open arms (Pellow *et al.*, 1985). Neurosteroids, such as allopregnanolone and THDOC, have equivalent effects on plus maze behaviour (Crawley *et al.*, 1986; Rodgers & Johnson, 1998).

The light-dark box also comprises an environment divided into aversive (light zone) and safe (dark zone) areas. As with the elevated plus maze, the test was validated using benzodiazepine anxiolytics (Crawley & Goodwin, 1980; Blumstein & Crawley, 1983), which release innate inhibition on mouse exploratory behaviour, and so increase exploration of both zones, especially the aversive light zone. Different investigators find divergent effects of anxiolytics on the various parameters scored within this paradigm, leading to debate over which parameters are the best measures of anxiety (e.g. see discussion by Hascoet & Bourin, 1998). Anxiolytic responses can include some or all of the following changes: increased time to first exit the light zone, increased time spent in the light zone, and an increase in exploratory locomotion within both zones. All of these parameters are therefore considered in this study.

5.1.3. $GABA_A$ receptor α2 subunits and neurosteroids in depression

Common mechanisms may underlie anxiety and depression, because these disorders are frequently co-morbid (Hirschfeld, 2001; Nutt *et al.*, 2006). One proposal is that deficiencies in GABAergic transmission could account for both disorders (see *Section 1.4.1*). Of particular note for this study, are observations that α2-/- mice have phenotypes consistent with both anxiety and depression (Dixon *et al.*, 2008; Vollenweider *et al.*, 2011) and that several limbic regions whose structure and/or activity is altered in depression, including the hippocampus, amygdala and basal ganglia (Sheline, 2003; McCabe *et al.*, 2009), are areas rich in $GABA_A$ receptor α2 subunit expression (Wisden *et al.*, 1992; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002; Mohler, 2006a). These data would support a role for α2-type $GABA_A$ receptors in depression.

Neurosteroids have also been linked with depression (see *Section 1.4.2*). The two key points being a negative correlation between endogenous allopregnanolone levels and depression symptoms in humans (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000), and that the antidepressant function of injected neurosteroids in animal models involves GABAergic signalling (Khisti *et al.*, 2000). We therefore propose that insufficient neurosteroid potentiation at $GABA_A$ receptor α2 subunits could underlie co-morbid anxiety and depression. This postulation was tested here by measuring depression-related behaviour in the α2^{Q241M} knock-in mouse strain. As with anxiety phenotyping, experiments examined baseline behaviour of wt and hom α2^{Q241M} mice, to expose any role for endogenous neurosteroids at α2-type $GABA_A$ receptors. Responses to injected THDOC were also measured, to elucidate any therapeutic potential for neurosteroids at these receptors.

In this study, depression-related phenotypes were screened using the tail suspension test paradigm. Mice are held upside-down by their tail for six minutes, during which time they fluctuate between two types of behaviour: periods of motion that reflect attempts to escape, and periods of immobility thought to represent 'behavioural despair'. This approach was developed in the 1980s (Steru *et al.*, 1985; Thierry *et al.*, 1986), and was validated by showing that known antidepressant drugs decreased behavioural despair (i.e. reduced 'immobility time') in mice. Increases in immobility time would therefore represent phenotypic depression. The tail suspension test was chosen in preference to the related forced swim test (Porsolt *et al.*, 1978) because it appears to be more sensitive to low doses of antidepressant, and does not induce hypothermic stress (see discussion in Thierry *et al.*, 1986).

5.2. Results

5.2.1. Endogenous neurosteroids act via $GABA_A$ receptor α2 subunits to determine basal anxiety levels

In both the elevated plus maze (*Fig. 5.1*) and light-dark box (*Fig. 5.2*) tests, hom knock-in mice have an anxiety-like phenotype under basal conditions. Anxiety inhibits the tendency of mice to explore novel environments, and is particularly manifest in reduced exploration of the aversive portions of the equipment used in these tests.

In the elevated plus maze hom animals spend significantly less time on the open arms than wt animals (*Fig. 5.1 A*), and tend to make fewer entries onto these arms (*Fig. 5.1 B*), although this latter trend does not reach significance ($p = 0.17$, paired t-test before correction for multiple comparison). Importantly, these effects are not explained by differences in activity within the maze: closed arm entries (*Fig. 5.1 C*) and total arm entries (*Fig. 5.1 D*) are invariant across genotypes. Heterozygous animals were included in this paradigm, and interestingly appear to have an anxiety phenotype intermediate between wt and hom animals, with data being neither significantly different to wt nor to hom mice.

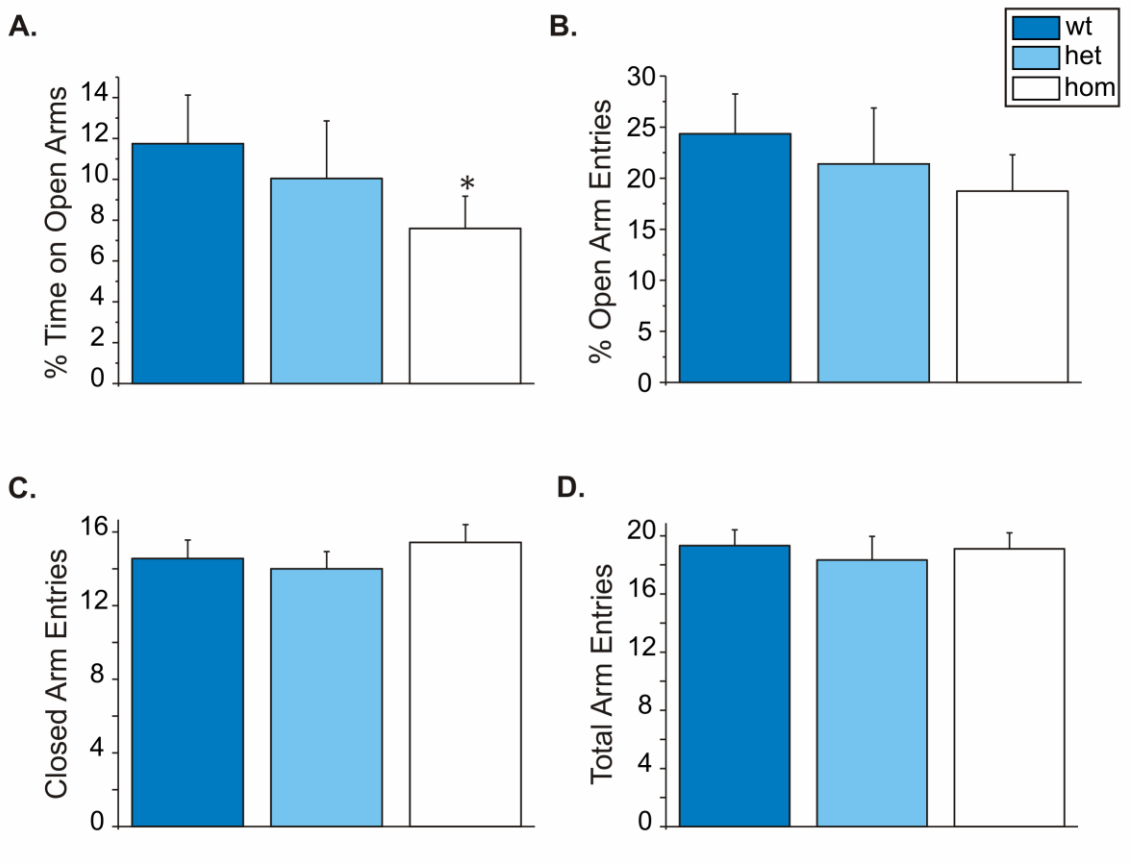

Figure 5.1 – α2^{Q241M} confers an anxious phenotype in the elevated plus maze

A. and B. Bar charts detailing the percentage time spent in the open arms (A) and the percentage of entries onto open arm (B) for each genotype (n=9 animals per genotype). There is a tendency for hom and het animals to spend less time on, and make fewer entries onto the open arms compared to wt. Hom animals spend significantly less time on open arms than wt littermates (* - $p<0.05$ paired t-test, Bonferroni corrected for multiple comparisons).

C. and D. Activity measures – total arm entries (C) and closed arm entries (D) – are unchanged across genotypes.

Elevated plus maze findings are corroborated by the behaviour of uninjected mice in the light-dark box paradigm. This test focussed on comparing wt and hom animals only. Several parameters scored in this procedure are consistent with hom mice being anxious relative to wt littermates. Firstly, hom mice leave the light zone more quickly at the start of the test than wt mice (*Fig. 5.2 A*). As well as this *active avoidance* response, anxiety in hom mice also increased their *passive avoidance* responses, manifesting in a decreased number of returns to

the light zone (i.e. fewer transitions between zones – *Fig. 5.2 B*) and a reduced time spent in the light zone (and correspondingly more time in the dark zone – *Fig. 5.2 C*).

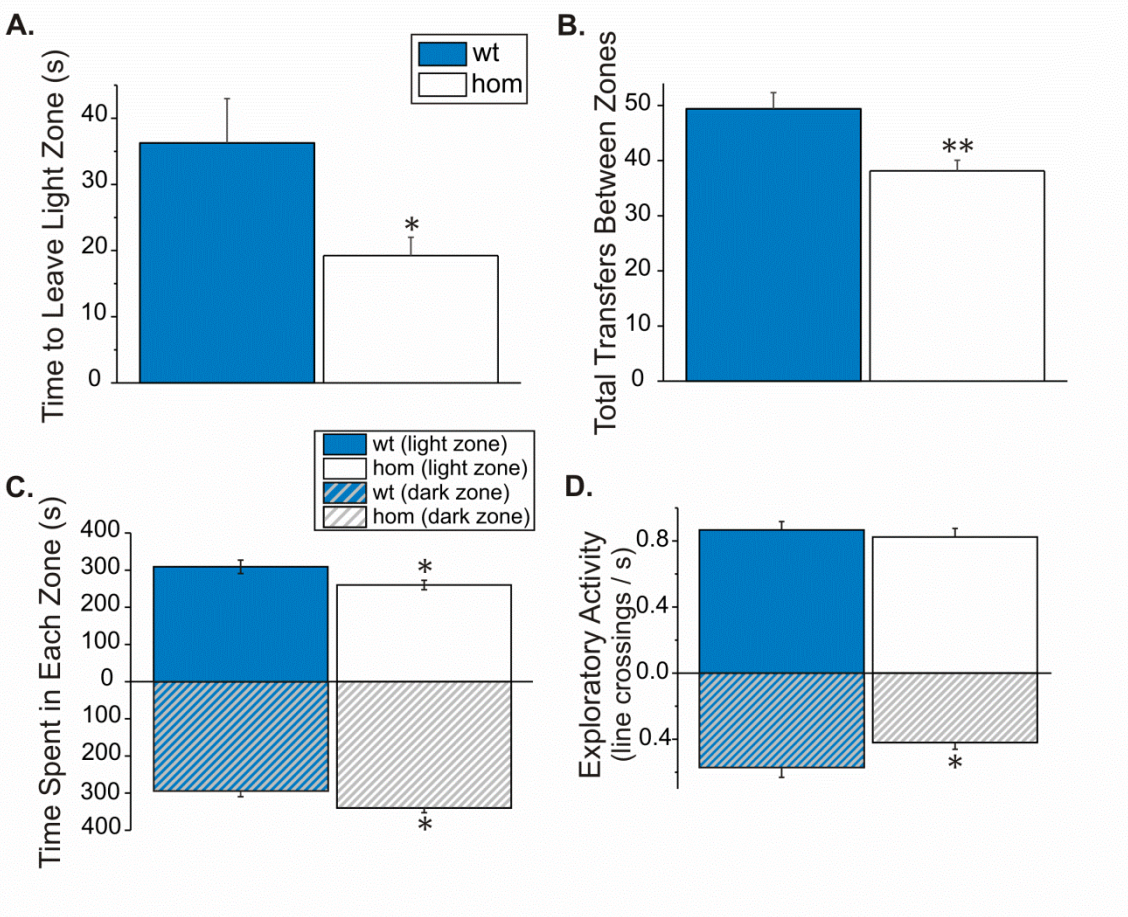

Figure 5.2 – α2^{Q241M} confers an anxious phenotype in the light-dark box

Homozygotes are faster to exit the light zone (A), make fewer transitions between the two zones (B), spend more time in the dark zone (C) and tend to show less exploratory activity (D) within the box than wt littermates (n=8 animals per genotype). Significant differences between genotypes are marked: * - p<0.05 and ** - p<0.01 (unpaired t-test).

The reduced exploratory activity of hom mice within the dark zone is also consistent with an anxiety phenotype (*Fig. 5.2 D*). Although the exploratory activity in the light zone is no different between wt and hom animals (*Fig. 5.2 D*), all of the other parameters are in line with an anxiety phenotype in hom animals.

Data from both tests therefore support the prediction that endogenous neurosteroids modulate baseline anxiety, at least in part, by potentiation at α2-type $GABA_A$ receptors.

5.2.2. $GABA_A$ receptor α2 subunits mediate the anxiolysis of injected neurosteroids at low doses

Having demonstrated a role for $GABA_A$ receptor α2 subunits in anxiolytic responses to endogenous neurosteroids, the investigation was extended to ask whether this subunit is also involved in mediating the anxiolytic response to exogenously applied neurosteroids. Plus maze and light-dark box tests were repeated, using mice pre-injected with the neurosteroid THDOC, at doses previously known to be anxiolytic in both tests (Wieland *et al.*, 1991; Rodgers & Johnson, 1998).

In the elevated plus maze, both doses of THDOC (10 mg/kg and 20 mg/kg) are anxiolytic in wt animals, increasing percentage time on open arms (*Fig. 5.3 A*) and percentage open arm entries (*Fig. 5.3 B*) relative to vehicle injected mice. The total number of arm entries increases at 20 mg/kg THDOC (*Fig. 5.3 D*), but there is no change in closed arm entries (*Fig. 5.3 C*). Although the former could suggest an increased locomotor activity, some investigators have suggested *closed arm entries* is a better activity measure, because it is independent of the (anxiety-related) number of open arm entries (Rodgers & Johnson, 1995).

THDOC injection is also anxiolytic in hom mice on the elevated plus maze, but only at the higher (20 mg/kg) dose: at 10 mg/kg THDOC, hom animals spend significantly less time on and make significantly fewer entries into open arms than wt littermates (*Fig. 5.3 A, B*), and these parameters are no different to those for vehicle-injected homozygotes. Such a rightward shift in the anxiolytic

dose-response curve for α2^{Q241M} mice is consistent with our prediction of reduced anxiolytic potency of neurosteroids.

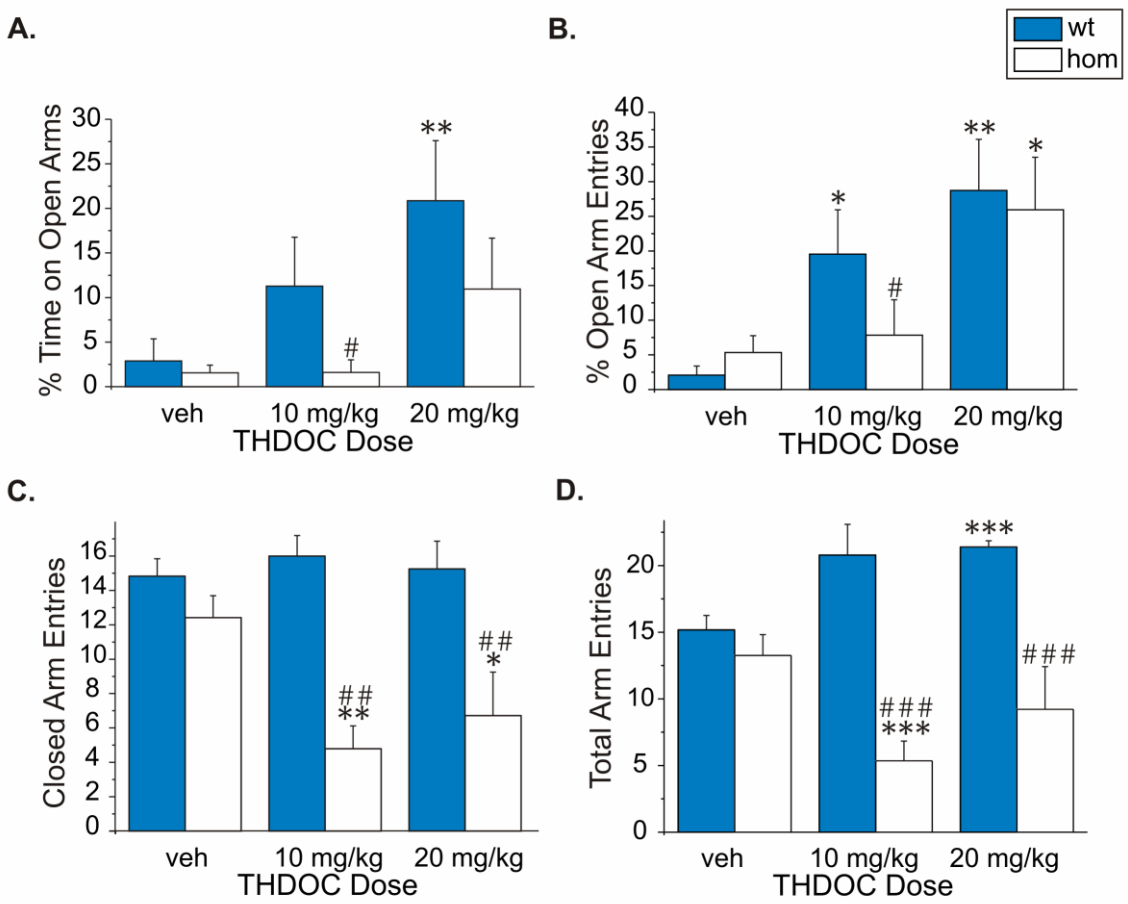

Figure 5.3 – α2^{Q241M} influences behavioural responses to injected THDOC on the elevated plus maze

A. and B. Bar charts detailing the effect of THDOC injection on percentage time spent on the open arms (A) and the percentage open arm entries (B) for each genotype.

C. and D. Activity measures – closed arm entries (C) and total arm entries (D) – are potently reduced by THDOC in hom animals only.

Data represent the mean (error bars, s.e.m.) of 6-7 animals per group. * - p<0.05, ** - p<0.01 and *** - p<0.001 for effect of THDOC vs. vehicle (veh); # - p<0.05, ## - p<0.01 and ### - p<0.001 for effect of genotype at a given dose of THDOC. Comparisons were either ANOVA, with multiple comparisons corrected with Benjamini-Hochberg adjustment (B, C), or all pairwise Behrens-Fisher comparisons (A, D).

Unexpectedly, hom animals respond to THDOC with profound reductions in locomotion: both doses significantly decreased closed arm entries (*Fig. 5.3 C*) and total arm entries (*Fig. 5.3 D*) when compared to vehicle treated animals, or to wt animals at equivalent THDOC dose. Whilst these activity effects could cast doubt over the genotypic difference in anxiety measures, the *percentage open arm entries* measure inherently accounts for total arm entries made during the test (i.e. the reduced activity is accounted for in *Fig. 5.3 B*). The conclusion that anxiolytic potency of neurosteroids is diminished in α2^{Q241M} mice therefore still stands. However, THDOC's ataxic effects pose some serious problems with the light-dark box approach, as will be discussed below. The mechanism underlying these activity effects is explored in *Section 5.2.5*.

In the light-dark box procedure, as with the elevated plus maze, injections of THDOC have classical anxiolytic effects in wt animals. The 20 mg/kg dose increases time to first exit the light zone compared to vehicle-treated animals (*Fig. 5.4 A*), consistent with an abated active avoidance response to the aversive light zone. This dose of THDOC also releases inhibition on exploratory activity in the wt mice, leading to increased transitions between the two zones (*Fig. 5.4 B*) and increased line crossings within each zone (*Fig. 5.4 D*). However, THDOC's anxiolytic effects are not manifest in an increased time spent in the light zone (*Fig. 5.4 C*).

Surprisingly, THDOC injection into hom mice has apparent anxiogenic effects in the light dark box, including significant reductions in transitions between zones (*Fig. 5.4 B*) and time spent in the light zone (*Fig. 5.4 C*). THDOC also tends to reduce exploratory activity in both zones; although the effect only reaches significance in the dark zone at 20 mg/kg vs. vehicle, activity is significantly lower than for wt animals at both doses of THDOC (*Fig. 5.4 D*). Given the potent ataxic effects of THDOC in hom animals within the elevated plus maze, however, it is difficult to determine whether these are truly anxiogenic effects, or an artefact of altered motor activity. For example, decreased time in the light zone may represent mice sleeping/resting in the dark zone, rather than avoiding the light zone.

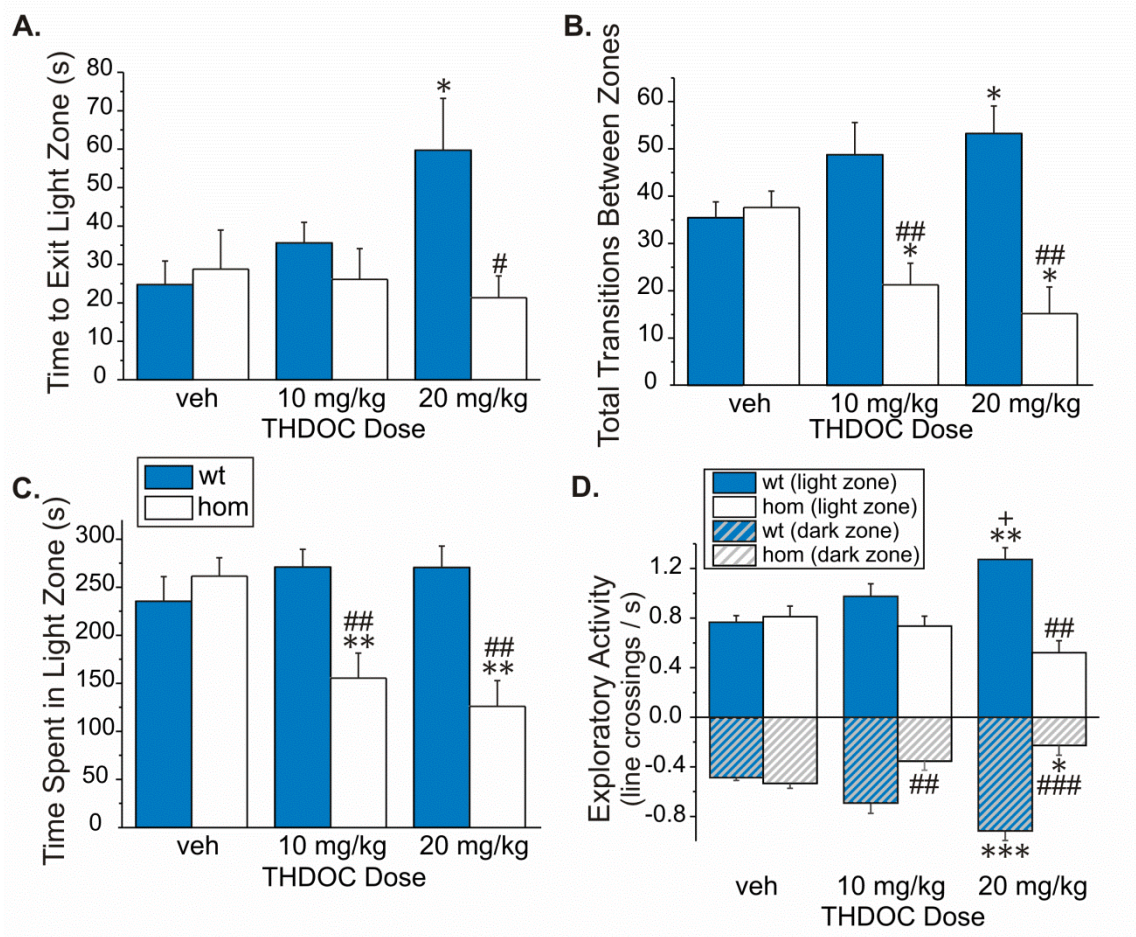

Figure 5.4 – α2^{Q241M} alters response to THDOC injection in the light-dark box

Bar charts detailing the effect of intraperitoneally-injected THDOC on time to exit the light zone (A), the number of transitions between the two zones (B), time spent in the light zone (C) and the exploratory activity within each zone (D).

Data represent the mean (error bars, s.e.m.) of 6-9 animals per group. * - $p<0.05$, ** - $p<0.01$ and *** - $p<0.001$ for effects of THDOC vs. vehicle (veh); + - $p<0.05$ for effect of 20 mg/kg vs. 10 mg/kg THDOC; # - $p<0.05$, ## - $p<0.01$ and ### - $p<0.001$ for effect of genotype at a given dose of THDOC. Comparisons were by ANOVA, with multiple comparisons corrected with Benjamini-Hochberg adjustment, except dark zone exploratory activity, which was subject to all pairwise Behrens-Fisher comparisons.

Unlike in the elevated plus maze, there is no easy means to correct for THDOC's activity effects in the light-dark box. Interestingly, THDOC-treated hom mice still exit the light zone very quickly (*Fig. 5.4 A*). Apparently, the initial need to escape from the aversive light zone is sufficient motivation for active avoidance, despite the drug's activity-reducing effect. Time to exit the light zone

at 20 mg/kg THDOC is no different to that for vehicle treated mice – consistent with a lack of anxiolytic effect of the drug in hom animals. Therefore, light-dark box results may well concur with those from the elevated plus maze.

Scoring for the light-dark box test was performed in two minute bins, allowing any time-dependent effects to be probed. Habituation of mice to the new environment of the box manifests in a tendency for reduced exploratory activity, in either zone, with increasing time. Habituation of wt and hom animals occurs at similar rates following vehicle injection (*Fig. 5.5 A*), but the effects of THDOC treatment on activity timecourses diverge between genotypes. THDOC counteracts habituation in wt mice (*Fig. 5.5 B*), and at the higher dose actually increases activity over time (compare bins 2/3 with bin 1). Conversely, THDOC accelerates habituation of hom mice (*Fig. 5.5 C*), with significant activity reductions occuring at earlier time bins than vehicle-treated controls.

Interestingly, THDOC does not significantly affect activity of either genotype in the first scoring bin (0-2 min timepoint of test, effects of dose: wt, $p = 0.20$; hom, $p = 0.22$). The time spent in each zone during this time bin may therefore be used as an anxiety measure without confound from activity effects (*Fig. 5.5 D*). A trend consistent with THDOC-mediated anxiolysis (increased time in light zone / decreased time in dark zone) is seen in wt animals, although this does not quite reach significance. Conversely, THDOC appears anxiogenic in hom animals (less time in light zone / more time in dark zone) with significant effects of 20 mg/kg THDOC vs. vehicle-treated homs and vs. 20 mg/kg THDOC-treated wt mice. When the same analysis is applied to data from uninjected animals (*Fig. 5.5 E*), hom mice still tend toward an anxious phenotype, although the difference between genotypes is not quite significant ($p = 0.09$, t-test). Data in *Fig. 5.5 D* therefore seem consistent with elevated plus maze findings – the α2^{Q241M} mutation has disrupted the anxiolytic response to injected neurosteroid.

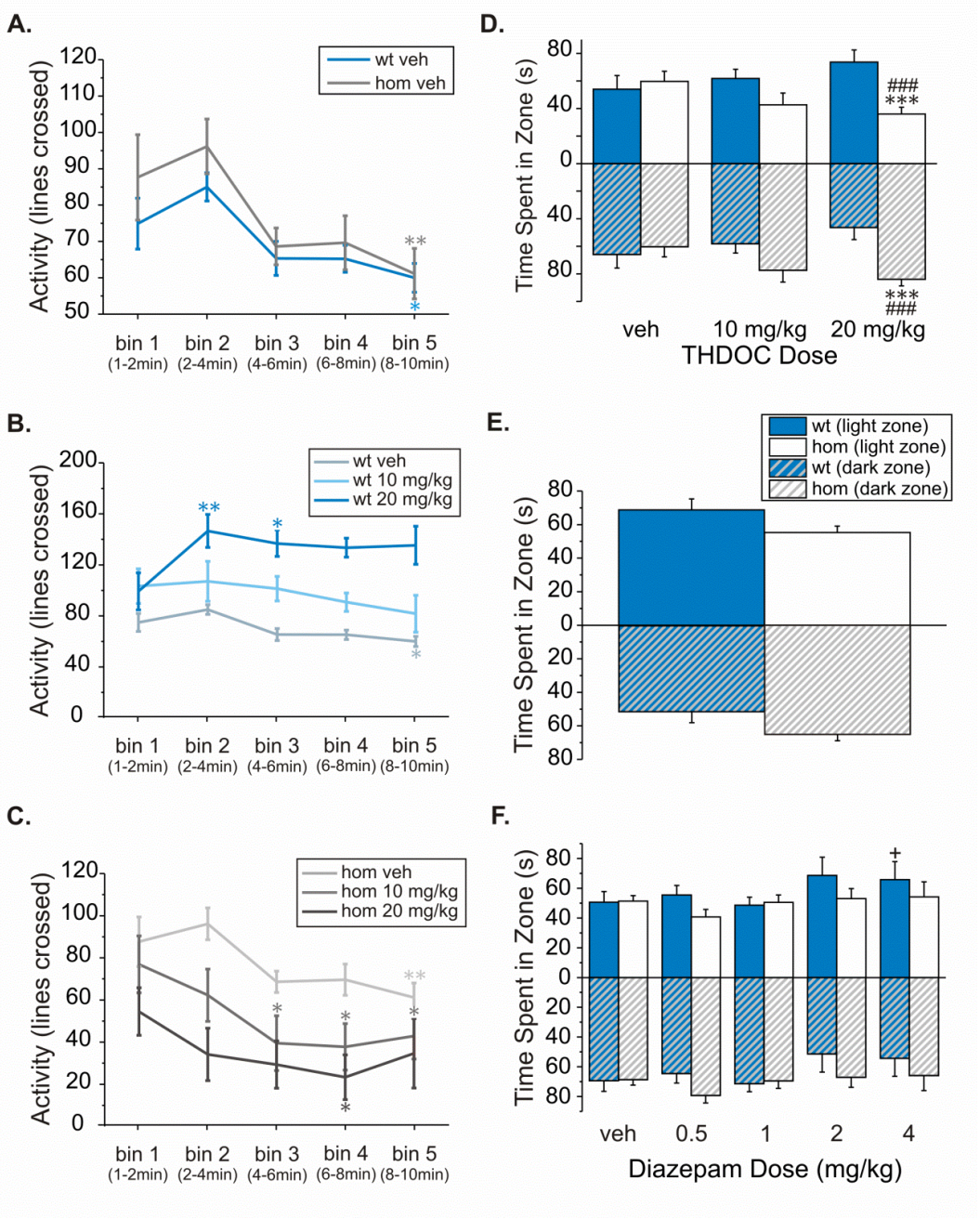

Figure 5.5 – Timecourse analysis of light-dark box results

A – C. Time courses for activity (grid lines crossed) in both zones of the light-dark box for mice injected with 2-hydroxypropyl-β-cyclodextrin vehicle (A) show a trend to decrease. This decrease is prevented by THDOC in wt animals (B), and accelerated by THDOC in hom animals (C). Activity within the first two minutes of the test (bin 1) is not significantly affected by THDOC in either genotype. Presented data represent the mean (error bars, s.e.m.) of 6-9 animals per group. * - $p<0.05$ and ** - $p<0.01$ (repeated measures ANOVA with Dunnett comparisons to bin 1).

D – F. Bar charts detail the time spent in each zone of the light-dark box in the first two minutes of the test for experiments with THDOC injected (D), untreated (E) and diazepam

injected (F) mice. Presented data represent the mean (error bars, s.e.m.) of 6-9 animals per group. *** - p<0.001 for effect of THDOC vs. vehicle (veh); + - p<0.05 for effect of 4 mg/kg diazepam vs. 0.5 mg/kg and 1 mg/kg doses of diazepam; ### - p<0.001 for effect of genotype at 20 mg/kg THDOC. Comparisons were either by ANOVA, with multiple comparisons corrected with Benjamini-Hochberg adjustment (F) or non-parametric all-pairwise Behrens-Fisher comparisons (D).

5.2.3. Anxiolytic effects of pentobarbital and diazepam are retained in α2^{Q241M} knock-in mice

Results presented above indicate a disrupted anxiolytic response to neurosteroids in α2^{Q241M} mice. This could be a specific consequence of losing neurosteroid potentiation at α2-type $GABA_A$ receptors, or due to some general defect in their anti-anxiety circuitry. Sensitivity to pentobarbital and diazepam potentiation is normal for α2^{Q241M}β3γ2S receptors expressed in HEK293 cells, and for sIPSCs recorded from brain slices of hippocampus and nucleus accumbens (*Fig. 3.1*, *Fig. 4.4*; Hosie *et al.*, 2006). A normal anxiolytic response to diazepam or pentobarbital would therefore be expected for α2^{Q241M} knock-in mice. However, refer back to *Section 3.3.3* for a discussion of how endogenous neurosteroids may complicate behavioural approaches. In spite of these complications, the anxiolytic potencies of pentobarbital and diazepam have been assessed using the plus maze and light-dark box procedures.

Pentobarbital was more potent in our mouse strain than reported elsewhere (Lister, 1987): in preliminary tests, 20 mg/kg pentobarbital induced loss of righting reflex in our strain. Lower intraperitoneal doses were therefore employed in this test (10 and 15 mg/kg). Although there is a significant increase in time spent on open arms for wt animals at 15 mg/kg (*Fig. 5.6 A*), this may well be a consequence of hyperactivity at this dose (increased total arm entries – *Fig. 5.6 D*). Using percentage open arm entries (*Fig. 5.6 B*) as an anxiety measure that is independent of altered activity, we see a trend to an increase

(i.e. anxiolysis) in both genotypes; however two-way ANOVA suggests the effect of drug is not quite significant (p = 0.06, F = 3.04, 2 d.f.). Encouragingly, we can be confident that there is no effect of genotype (ANOVA p = 0.32, F = 1.02, 1 d.f.), nor an interaction effect (ANOVA p = 0.64, F = 0.45, 2 d.f.). There is no effect of drug or genotype on activity as assessed by closed arm entries (*Fig. 5.6 C*), although there is a tendency towards increased activity at the higher dose of pentobarbital.

Because pentobarbital also appeared to be strongly motor impairing at the 15 mg/kg anxiolytic dose – mice showed an uncoordinated gait, or even fell off the open arms of the maze – this drug was not taken forward to light-dark box comparisons. Overall, elevated plus maze results suggest that pentobarbital tends to be anxiolytic with similar potency in both genotypes.

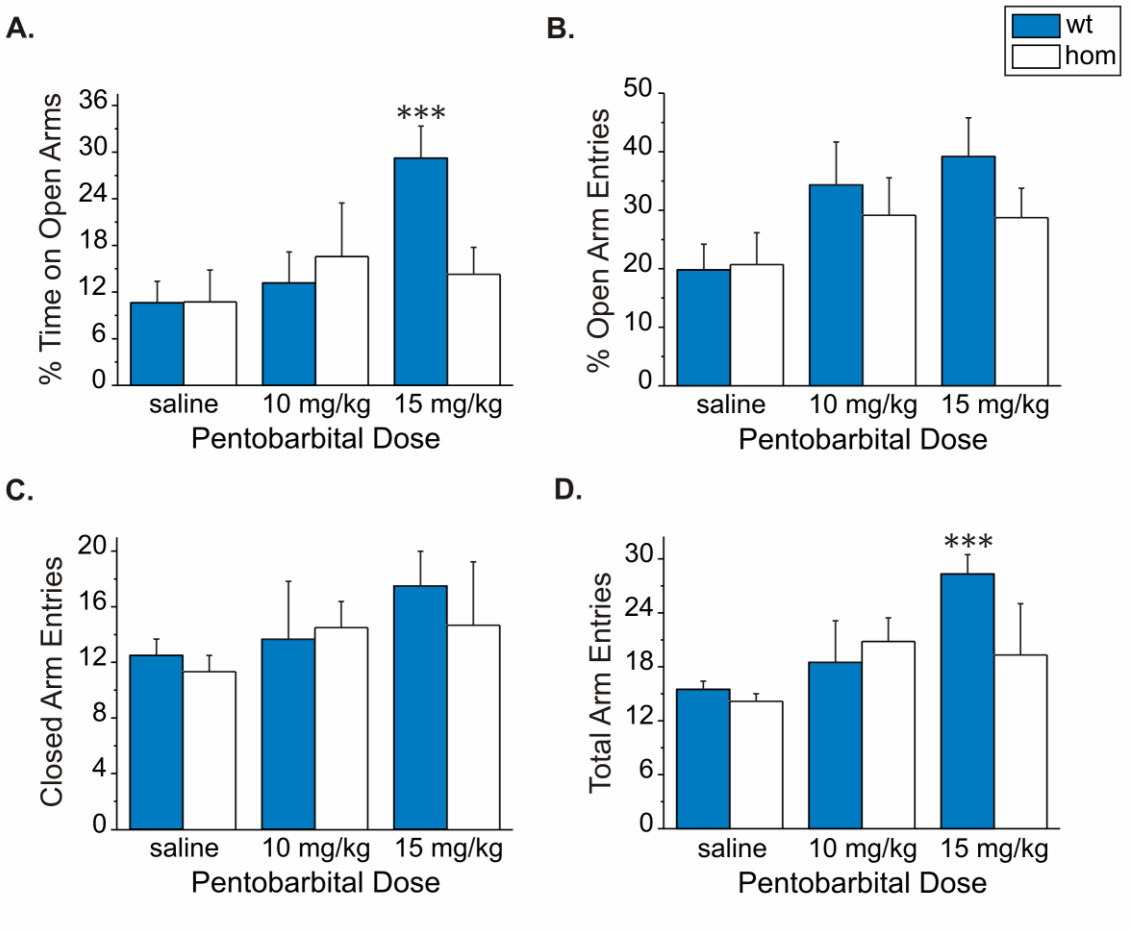

Figure 5.6 – α2^{Q241M} does not influence pentobarbital's effect on elevated plus maze behaviour

A. and B. Bar charts detailing the effect of pentobarbital injection on percentage time spent in the open arms (A) and the percentage open arm entries (B) for each genotype.

C. and D. Activity measures – closed arm entries (C) and total arm entries (D) – reveal pentobarbital-induced hyperactivity in wt animals only.

Data represent means (error bars, s.e.m.) for 6 animals per group. *** - p<0.001 for effects of drug vs. saline (all pairwise Behrens-Fisher comparisons).

Diazepam's anxiolytic effects in the elevated plus maze were seen in both wt and hom mice, which tend to spend more time on open arms with diazepam treatment (*Fig. 5.7 A*). For percentage time on open arms, two-way ANOVA finds an effect of drug (p = 0.02, F = 4.37, 2 d.f.) but no effect of genotype (p = 0.61, F = 0.27, 1 d.f.) and no interaction effect (p = 1.0, F = 0, 2 d.f.); none of the individual pairwise comparisons reach significance. Anxiolytic effects of

diazepam are additionally seen as increased percentage entries onto open arms (overall Kruskal-Wallis test, p = 0.03), individual pairwise comparisons in this case also reveal significant effects of drug vs. vehicle (and no differences between genotypes at any dose – see *Fig. 5.7 B*). There are no statistically significant effects of diazepam or genotype on activity measures within the plus maze (*Fig. 5.7 C, D*). Results are therefore consistent with a retained anxiolytic effect of diazepam in α2^{Q241M} mice.

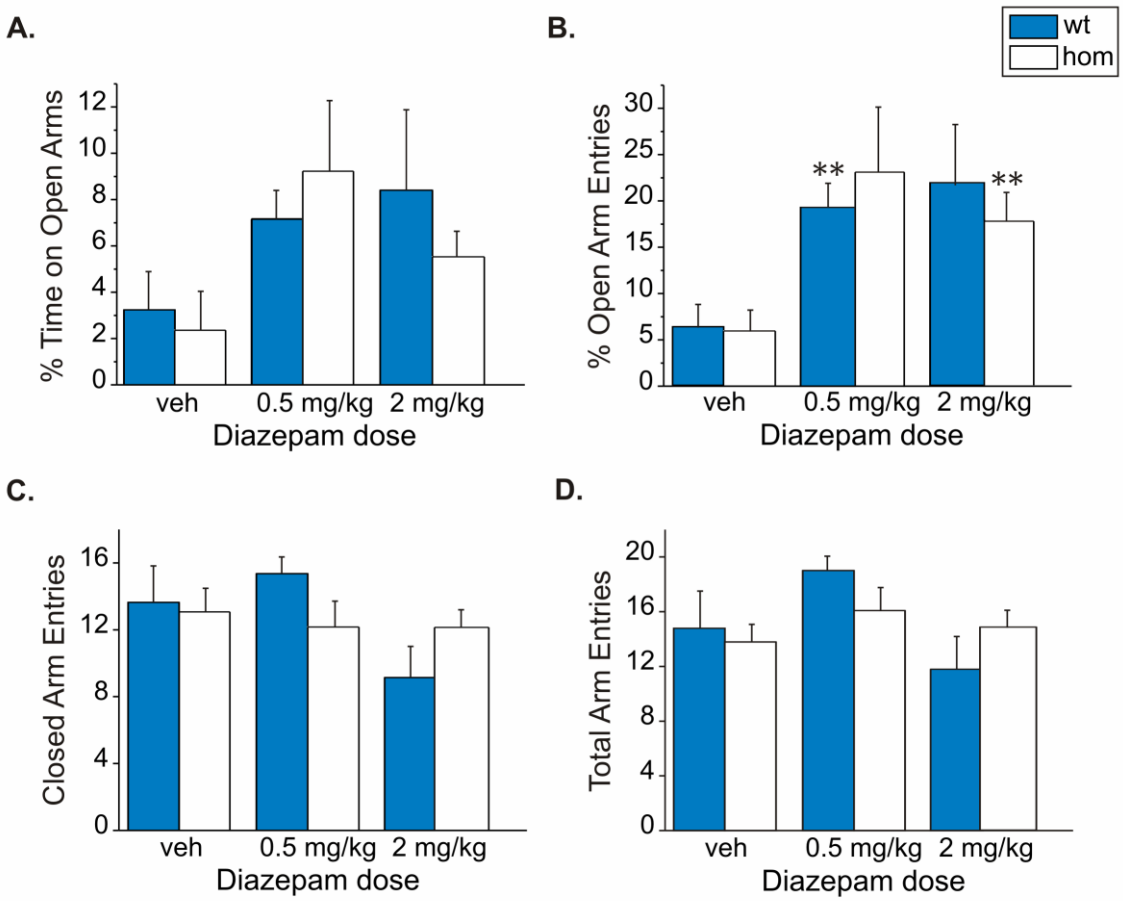

Figure 5.7 – α2^{Q241M} does not influence diazepam's effect on elevated plus maze behaviour

A. and B. Bar charts detailing the effect of diazepam injection on percentage time spent in the open arms (A) and the percentage open arm entries (B) for each genotype.

C. and D. Activity measures – closed arm entries (C) and total arm entries (D) – are not significantly affected by genotype or diazepam administration.

Data are the mean (error bars, s.e.m.) of 6-9 animals per group. ** - p<0.01 for effects of diazepam vs. vehicle (veh) (all pairwise Behrens-Fisher comparisons).

A wider range of diazepam doses was employed in the light-dark box procedure to probe dose-responses in finer detail. Diazepam's effects in wt animals closely mirror the pattern of effects seen by Hascoet and Bourin (1998). We see a biphasic effect of diazepam on activity in light and dark zones (*Fig. 5.8 D*): low doses, 0.5 and 1 mg/kg, tend to increase activity (anxiolytic effect), whilst higher doses, 2 and 4 mg/kg, tend to decrease activity (sedative effect). Although increased time to exit the light zone at 2 and 4 mg/kg (*Fig. 5.8 A*) could represent anxiolytic effects of the drug, decreased exploratory activity at these doses implicates a contribution of sedation. Transitions between zones (*Fig. 5.8 B*) and time spent in the light zone (*Fig. 5.8 C*) fail to demonstrate diazepam anxiolysis. The former parameter only revealed sedative effects of higher diazepam doses, and the latter shows no significant variation with dose.

Homozygous animals appear unresponsive to diazepam in the light-dark box test, showing no increase in time to exit light zone (*Fig. 5.8 A*), no change in transitions between zones (*Fig. 5.8 B*), no change in time spent in the light zone (*Fig. 5.8 C*), nor any alteration in exploratory activity in either zone (*Fig. 5.8 D*). Exploratory activity clearly depends on a mixture of anxiolysis (increases activity) and sedation (decreases activity). The apparent diazepam insensitivity of exploratory activity in hom animals could result if these mice were more sensitive to sedation – e.g. sedation at a dose of 1 mg/kg cancelling out the activity increase expected for anxiolysis. This notion is consistent with the apparent hom hypersensitivity to sedation with THDOC. However, *transitions between zones* and *time to exit light zone* results are not consistent with 'hyper-sedation' by diazepam, arguing instead that hom α2^{Q241M} mice are simply insensitive to diazepam in this test. Furthermore, even when focussing only on the first time bin of scoring (*Fig. 5.5 F*), an approach that helped to reduce the problem of THDOC's activity effects, data still fail to demonstrate an anxiolytic effect of diazepam.

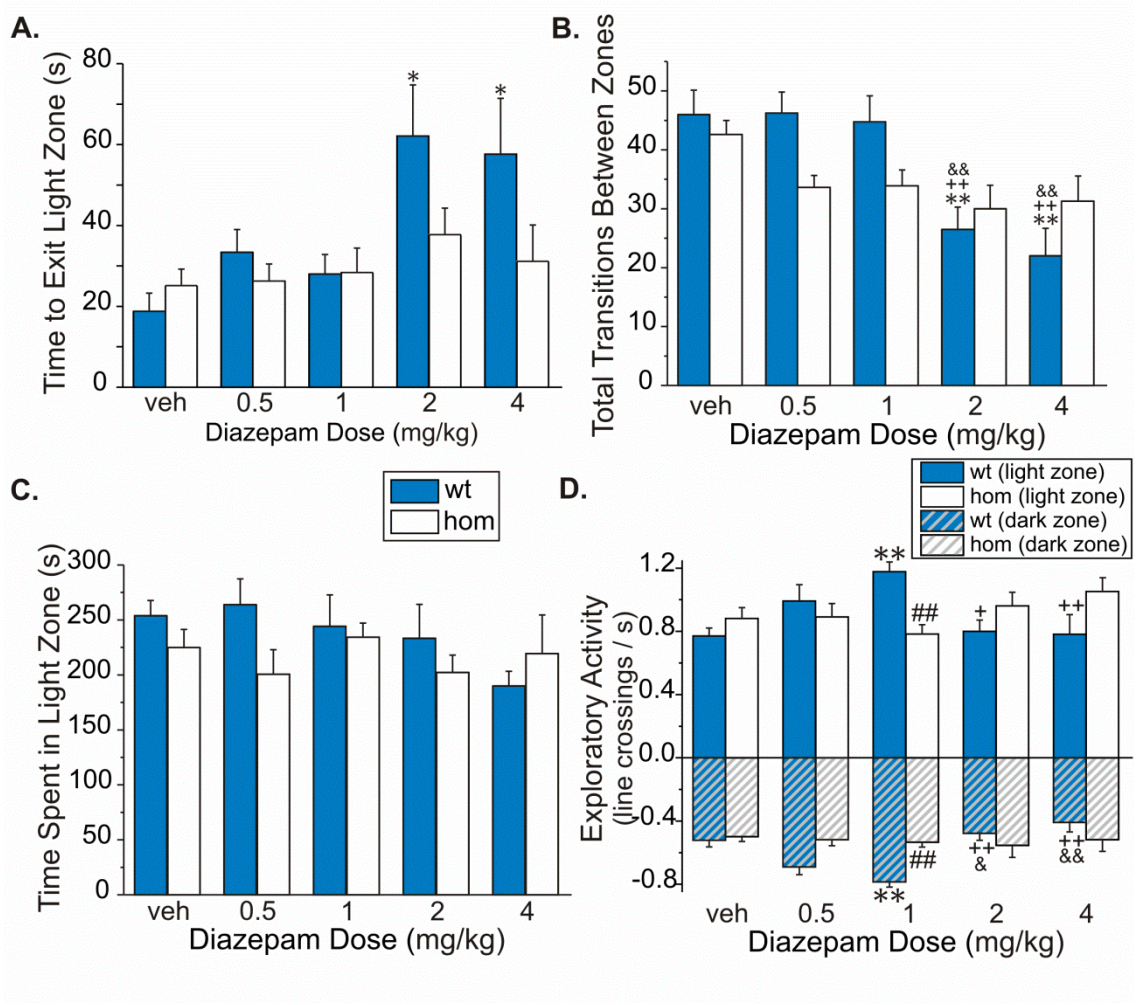

Figure 5.8 – α2^{Q241M} alters response to diazepam in the light-dark box

Bar charts detailing the effect of intraperitoneally-injected diazepam on time to exit the light zone (A), time spent in the light zone (B), the number of transitions between the two zones (C) and the exploratory activity within each zone (D).

Presented data represent the mean (error bars, s.e.m.) of 7-8 animals per group. Statistically significant differences are highlighted: * - $p<0.05$ and ** - $p<0.01$ for effect of diazepam dose vs. vehicle (veh); + - $p<0.05$ and ++ - $p<0.01$ for effect of diazepam dose vs. 1 mg/kg diazepam; & - $p<0.05$ and && - $p<0.01$ for effect of diazepam dose vs. 0.5 mg/kg diazepam; # - $p<0.05$ and ## - $p<0.01$ for effect of genotype at a given diazepam dose. Comparisons were by ANOVA, multiple comparisons corrected by Benjamini-Hochberg adjustment.

5.2.4. Knock-in mutation α2^{Q241M} does not confer a depression phenotype

There is no effect of the knock-in mutation on the immobility time of untreated mice subjected to the tail suspension test (*Fig. 5.9 A*), which suggests that the α2^{Q241M} mutation does not induce depression – at least not in the homozygous state. Injected THDOC has no effect on immobility time of wt mice, and tends to increase immobility in hom mice at both doses (*Fig. 5.9 B*). Two-way ANOVA results indicate an effect of genotype (p = 0.006, F = 8.92, 1 d.f.), no overall effect of THDOC treatment (p = 0.061, F = 3.08, 2 d.f.) and no interaction effect (p = 0.316, F = 1.2, 2 d.f.). Unadjusted LSD comparisons suggest significant effects of 5 mg/kg (p = 0.03) and 10 mg/kg (p=0.03) THDOC vs. vehicle for hom animals, and a significant difference between immobility time for wt and hom animals at 10 mg/kg THDOC (p = 0.01); however, none of these values survive correction for multiple comparisons.

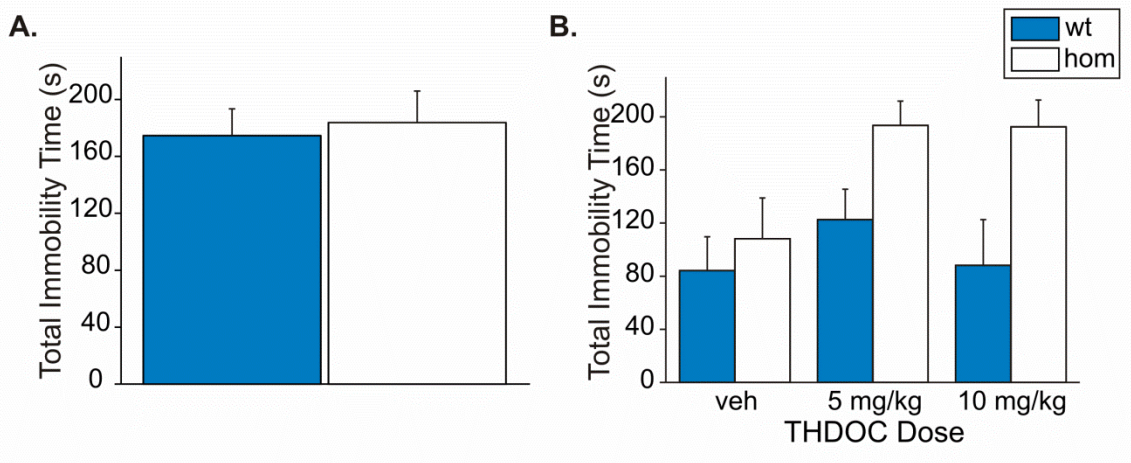

Figure 5.9 – Immobility in the tail suspension test is unaffected by mutation α2^{Q241M}

A. Baseline behavioural despair (total immobility time) in untreated animals is no different across genotypes (n=8 animals per genotype).

B. THDOC does not decrease immobility time in wt animals; the trend to increased immobility time with THDOC dose in hom animals is consistent with ataxic effects observed in other tests. None of the individual pairwise comparisons reach significance (n=5-7 animals per group).

The tendency toward increased immobility in hom animals is consistent with above observations that THDOC reduces motor activity in these mice, and so is unlikely to indicate a depressive effect of THDOC injection. The lack of reduction in immobility in wt mice suggests that THDOC is not antidepressant within the tail suspension test.

5.2.5. Ataxic effects of THDOC injection in hom mice: not a consequence of increased motor impairment

Performance of wt and hom mice on an accelerating rotarod was used as a measure of motor coordination. Scoring assessed the time taken for the mice to fail the test by either falling off the rod, or passively rotating with it. As can be seen from *Fig. 5.10 A*, initial performance on the rod is no different between wt and hom mice – i.e. the α2^{Q241M} mutation has no inherent motor-impairing effects.

Mice were next trained to achieve a consistent and reproducible performance on the rotarod, as detailed in the *Section 2.7.5*. Performance of these mice was then assessed following THDOC injection, and expressed for each mouse relative to his performance on the preceding training day (*Fig. 5.10 B*). There was no motor impairing effect of vehicle or THDOC at a dose of 10 mg/kg. An approximately 20 percent decline in performance is seen for both wt and hom animals at 20 mg/kg THDOC. Whilst the effect of drug only reaches significance for hom animals, there is no difference in performance of wt and hom animals at any dose. In addition, 10 mg/kg THDOC, which has clear activity-reducing effects in hom animals in other behavioural tests, produces no motor impairment within the rotarod test. This would suggest that the activity effects of THDOC cannot be explained by an enhancement of motor impairing effects of this drug in hom mice.

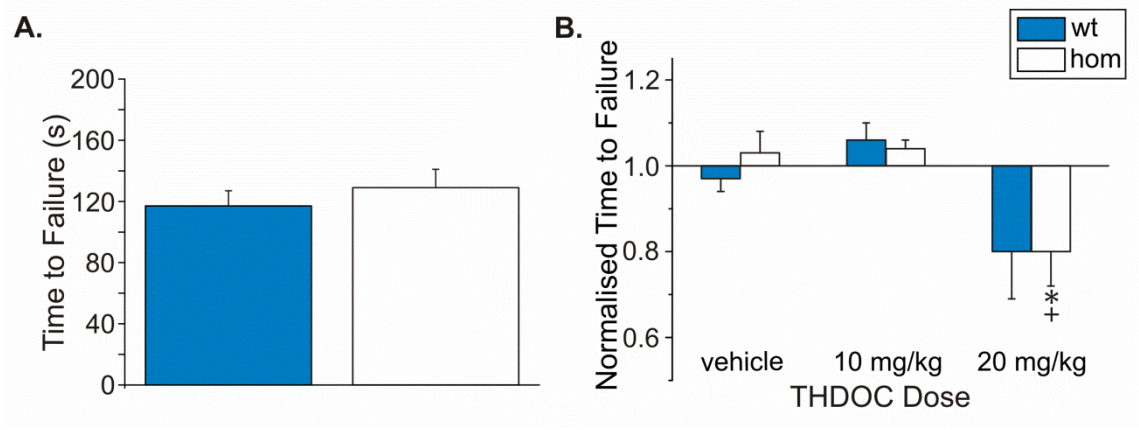

Figure 5.10 – α2^{Q241M} does not affect performance on the accelerating rotarod under baseline or THDOC-treated conditions

A. Baseline performance on the rotarod (time to fail the test) is no different across genotypes.

B. THDOC impairs motor performance on the rotarod (reduces time to failure) only when administered at a dose of 20mg/kg. This effect reaches significance in hom animals: * - $p<0.05$ for effect of 20 mg/kg THDOC vs. vehicle (veh); + - $p<0.05$ for effect of 20 mg/kg THDOC vs. 10 mg/kg THDOC; all pairwise Behrens-Fisher comparisons.

5.3. Discussion

5.3.1. Neurosteroids act via α2-type GABA$_A$ receptors to modulate anxiety state

Two behavioural screens for anxiety – the elevated plus maze and the light-dark box – both demonstrate an anxious phenotype for untreated hom α2^{Q241M} mice relative to wt littermates. This result is consistent with a model in which endogenous neurosteroid tone in a naïve untreated mouse regulates its baseline anxiety state through α2-type GABA$_A$ receptors. This conclusion relies on a lack of compensatory changes that may provide an alternative explanation for these behavioural differences. In favour of this, no changes in GABA$_A$ receptor protein expression were detected for subunits α1-α5 (see *Chapter 3*).

Heterozygous animals were included in the elevated plus maze paradigm, and interestingly appear, by trend, to have an anxiety phenotype intermediate between wt and hom animals. This appears incongruous with work in brain slices, where THDOC-mediated prolongation of sIPSC decay time is the same in cells from het and wt animals (*Fig. 4.3*, *Fig. 4.4*). However, het mice have faster decaying baseline sIPSCs than wt mice (*Table 4.1*), suggesting the α2^{Q241M} mutation has some effect when expressed in the heterozygous state. As described in *Section 4.3.1*, a single neurosteroid potentiation site is believed sufficient to achieve the full potentiating effect of neurosteroid, and so only a quarter of the α2-containing pentamers in het animals should be neurosteroid insensitive. Perhaps this small deficit is sufficient to produce a phenotypic effect in some situations. Nevertheless, subsequent experiments focussed on comparing only wt and hom animals, where we can be sure that neurosteroid potentiation has been ablated at all α2-type GABA$_A$ receptors in the knock-in mice.

Anxiolytic effects of injected neurosteroids are well established (Crawley *et al.*, 1986; Wieland *et al.*, 1991), and the stress-induced rise in neurosteroids has

been purported to be a natural anxiolytic response to restore GABAergic tone after stress (Purdy *et al.*, 1991; Barbaccia *et al.*, 1998). Here we provide the first direct evidence for endogenous neurosteroids having an anxiolytic effect in an unperturbed animal, via α2-type $GABA_A$ receptors.

Our suggestion that α2-type $GABA_A$ receptors may be mediators of the anxiolytic response to injected neurosteroid is also supported by the results of behavioural screening. For the elevated plus maze, a clear rightward-shift in the dose-response curve for *percentage open arm entries* indicates a defective anxiolytic response to THDOC in hom mice. The anxiolytic response to the higher dose (20 mg/kg THDOC) does not invalidate our model: α2 and α3 isoforms appear to have overlapping roles in benzodiazepine-mediated anxiolysis and myorelaxation, with α2 mediating low-dose effects, and α3 mediating high-dose effects (see *Section 1.3.2*). We may be observing a related phenomenon for THDOC-mediated anxiolysis (i.e. 10 mg/kg acts via α2 subunits, whilst 20 mg/kg can act via α3 subunits). Future experiments to confirm this hypothesis would be to cross-breed α2^{Q241M} mice with either α3 knock-out mice (e.g. those generated by Yee *et al.*, 2005) or neurosteroid-insensitive α3^{Q266M} knock-in mice. Mice resulting from such crosses would lose neurosteroid potentiation at both α2 and α3 isoforms of the $GABA_A$ receptor, and be predicted to demonstrate no anxiolytic response to injected THDOC, even at high doses.

Modulation of non-GABAergic transmission could also account for the residual anxiolysis in hom α2^{Q241M} mutants. Whilst $GABA_A$ receptors are sensitive to nanomolar levels of neurosteroids, micromolar levels can also modulate glutamate (NMDA, AMPA and kainate), glycine, serotonin, nicotinic acetylcholine, oxytocin, and sigma-type-1 receptors (see review by Rupprecht & Holsboer, 1999). It is difficult to estimate what brain concentration of THDOC results from our intraperitoneal injections, which will depend on a number of factors. Relative absorption and clearance rates will determine the pharmacokinetic profile, whilst a number of factors – such as propensity to

cross the blood-brain barrier – will influence the relative distribution in the brain vs. other tissues. In rats, 30 min following subcutaneous injection of 8 mg/kg allopregnanolone, brain levels of this compound reach 400 ng per gram tissue (Vallee *et al.*, 2000). If intraperitoneally injected THDOC were to act similarly in our mouse strain, and if the amount reaching the brain were directly proportional to the amount injected, a 20 mg/kg dose of THDOC might be expected to produce a brain level of approximately 1000 ng/g tissue (i.e. 3 micromoles per kg tissue). Whilst this extrapolation is based on some oversimplifying assumptions, it would suggest that brain THDOC concentrations in injected mice could be in the range of the micromolar levels that modulate non-GABAergic transmission.

THDOCs anxiolytic effects in wt mice subjected to a light-dark box test have been reported previously (Wieland *et al.*, 1991). We see this effect here as an increased time to exit the light zone, increased activity in both zones and increased transitions between zones. The time spent in the light does not significantly increase, but as alluded to earlier (*Section 5.1.2*), different investigators report distinct patterns of drug effects on light-dark box parameters. For example, no effect of diazepam on this parameter has been observed by Hascoet and Bourin (1998), depending on mouse strain and precise conditions of the test. Perhaps under some testing conditions, vehicle-injected mice are already spending maximal time in the light zone, precluding a further increase by an anxiolytic.

Drug effects on activity measures complicate matters further. For example, not all investigators agree with using increased *time to exit light zone* as an indication of anxiolysis because it could also indicate sedation. Given that 20 mg/kg THDOC increases wt exploratory activity, the increased time to exit the light zone probably represents anxiolysis rather than sedation. The increased wt activity following THDOC injection (increased transitions between zones and increased exploratory activity) could also represent a stimulant effect of

THDOC, but the lack of increase in *closed arm entries* on the elevated plus maze indicates anxiolysis is the more likely explanation.

THDOC has potent ataxic effects in hom mice, precluding a standard light-dark box approach to studying anxiety, because it is difficult to disentangle activity and anxiety effects within this test. There are some indications, however, that α2^{Q241M} knock-in disrupts anxiolysis. Firstly there is no increase in time to exit the light zone with THDOC treatment of hom mice (*Fig. 5.4 A*), whilst activity-reducing and anxiolytic effects of this drug would both be expected to increase this duration. It would therefore seem that placement in the light zone is sufficiently aversive to motivate an active avoidance response in hom mice, despite the activity-reducing effects of THDOC. Additional support for failure of anxiolysis in hom mice comes from analysis of the first two minutes of the test, where activity effects are not significant. Using the relative time spent in light vs. dark zones as an anxiety measure, wt mice show an anxiolytic-like response to THDOC, but hom mice demonstrate anxiogenesis. Whilst anxiogenesis was not seen in the elevated plus maze, these data concur that α2^{Q241M} has disrupted anxiolysis.

The tests used in this body of work probe only one type of anxiety, relating to inhibition of exploratory behaviour. This anxiety can also be probed with an open field apparatus, but one would predict results to be very similar to those of the light-dark box test – and to suffer the same difficulties when trying to distinguish emotionality from locomotor/activity effects (Stanford, 2007). Alternative rodent anxiety screens include conditioned paradigms, where subjects learn to associate particular non-noxious environmental cues with a co-presented foot-shock, and subsequently demonstrate fearful responses to these cues in the absence of foot-shock. There is a precedent for a role of $GABA_A$ receptor α2 subunits in this type of anxiety, because α2-/- mice show an anxious phenotype in a conditioned emotional response task (Dixon *et al.*, 2008). It would be interesting to compare α2-/- and hom α2^{Q241M} mice in such a task, to investigate whether the anxious phenotype of α2-/- mice is a

consequence of losing neurosteroid potentiation at the α2-type $GABA_A$ receptors.

5.3.2. Anxiolytic effects of pentobarbital and diazepam

To ensure that the disrupted anxiolysis in hom α2^{Q241M} mice is purely a consequence of lost neurosteroid potentiation at the α2-type $GABA_A$ receptors, and not a general inability of hom mice to respond to any anxiolytic agent, behavioural studies were extended to include other $GABA_A$ receptor potentiators. If the normal circuitry for anxiety is intact in hom mice, and there have been no compensatory changes in response to the α2^{Q241M} mutation, sensitivity to diazepam and pentobarbital should be retained. This is certainly true for the electrophysiological effects of these drugs when the mutation is studied in HEK cells or brain slices from the transgenic mice (*Fig. 3.1*, *Fig. 4.4*; Hosie *et al.*, 2006).

Pentobarbital's anxiolytic effects are not particularly potent in either genotype in our mouse strain. For percentage time spent in open arms, it seems that there is a tendency for an overall effect of drug, but no overall effect of genotype, which would be consistent with retained anxiolytic function of pentobarbital in α2^{Q241M} mutant mice. Lister (1987) demonstrated pentobarbital anxiolysis in the elevated plus maze, but only at a dose of 30 mg/kg; it was not possible to try this dose in our mouse strain because preliminary tests with this drug found loss of righting reflex at 20 mg/kg. Pentobarbital was not extended to the light-dark box paradigm due to its effects on activity, and because, the anxiolytic dose of pentobarbital (15 mg/kg) appeared, anecdotally, to be motor-impairing in animals of both genotypes.

Diazepam effects on wt animals are as one would expect for an anxiolytic drug in both behavioural screens, although not all parameters in the light-dark box

are consistent with anxiolysis. As expected exploratory activity rises at low doses (anxiolysis) and falls at higher doses (sedation). A lack of increase in time spent in the light zone might be expected, given that THDOC also fails to increase this parameter in wt mice. Unlike with THDOC, when examining the 'transitions between zones' parameter, diazepam-treated mice also fail to show anxiolysis, only revealing sedative effects of higher doses. Methodological differences between tests using THDOC and diazepam, such as the post-injection time at which mice are tested (15 min and 30 min, respectively), may account for this difference. Mice treated with diazepam-vehicle are less anxious than the THDOC-vehicle mice (46 ± 4 vs. 36 ± 3 transitions, respectively). A lower baseline anxiety level for the diazepam-dose response curve reduces scope for anxiolysis to be detected for this parameter (analogous to the time spent in the light zone).

In this study, spatiotemporal anxiety measures within the elevated plus maze show similar anxiolytic effects for diazepam in wt and hom mice. It is unclear why hom mice fail to display a response to diazepam in the light-dark box. The latter test is more susceptible to any activity differences between groups being compared, and there may be some subtle genotypic differences in activity effects that are precluding observation of anxiolysis. One might therefore place more faith in the elevated plus maze paradigm, in which activity effects can be accounted for before making conclusions about anxiety. On that basis, it appears that there is no clear effect of the mutation on diazepam sensitivity, and the loss of THDOC sensitivity is a specific effect of the α2^{Q241M} mutation. To support this conclusion, it would be sensible to repeat the diazepam dose-response analysis on the plus maze, using the wider range of doses that was employed in the light-dark box test, to examine the anxiolytic dose-response curve in finer detail.

One must also remember that responses to injected drugs are taking place on a background of endogenous neurosteroids. Because the response to these endogenous steroids is diminished in hom mice, we might also expect a

disrupted response to diazepam (see *Section 3.3.3*). Adding further complexity, diazepam can increase the production of these endogenous neurosteroids, via the 'peripheral benzodiazepine receptor' (now called translocator protein 18, TSPO (Papadopoulos & Lecanu, 2009)). Interestingly, accumulating evidence implicates this neurosteroid production as a mediator in the overall *in vivo* response to benzodiazepines (e.g. midazolam's anticonvulsant effect is partly mediated via neurosteroid production (Dhir & Rogawski, 2012)). The lack of diazepam-mediated anxiolysis we observe for hom mice in the light-dark box may therefore be suggesting a role for endogenous neurosteroids in this response. To probe whether this is the case, mice could either be pre-treated with finasteride (to block neurosteroidogenesis), or the experiment repeated with a benzodiazepine that does not act on TSPO, e.g. zolpidem (Trapani *et al.*, 1997). The former would be more challenging to implement, as measurements would need to confirm that finasteride has successfully ablated production of endogenous neurosteroids, but would probably be preferable to the latter (since endogenous neurosteroids other than those produced by benzodiazepine action at TSPO could still account for a difference in anxiolysis by zolpidem).

5.3.3. Depression may not involve neurosteroids acting at α2-type GABA$_A$ receptors

Anxiety and depression are frequently co-morbid, both may be treated with SSRIs and probably share common underlying patho-physiological mechanisms (Hirschfeld, 2001; Nutt *et al.*, 2006; Smith & Rudolph, 2012). The depressed phenotype of α2-/- mice (Dixon *et al.*, 2008), and involvement of neurosteroids in depression (discussed in *Section 1.4.2*) may lead one to predict that hom α2^{Q241M} mice will be depressed as well as anxious at baseline. However, this is not what we observe using the tail suspension test (*Fig. 5.9 A*). Nevertheless, a single test cannot discount a phenotype; for example α2-/- mice were judged 'depressed' in most, but not *all* behavioural tests tried (Vollenweider *et al.*, 2011). Furthermore, heterozygous α2 knockout (α2+/-) mice often showed a

phenotype that was absent in homozygous knockouts (α2-/-), possibly due to (undefined) compensatory changes in α2-/- mice that reduce depression which are not present in the α2+/- mice. Perhaps the same is true of α2^{Q241M} knock-in mice; future experiments should therefore not only consider alternative tests for depression, but also include het mice. Alternative screens for depression include 'novelty suppressed feeding' (Dulawa & Hen, 2005), forced swim (Porsolt *et al.*, 1978) and sucrose-preference (Papp *et al.*, 1991) tests. If α2^{Q241M} mice fail to show a phenotype in these tests, anxiety and depression may well be less tightly coupled than currently thought, with anxiety more closely linked with neurosteroid regulation of α2-type $GABA_A$ receptors. Consistent with anxiety and depression being dissociable phenomena, $GABA_B$ receptor knockout mice are more anxious but *less* depressed than wt counterparts (Mombereau *et al.*, 2004; Mombereau *et al.*, 2005).

Surprisingly, THDOC does not appear to be antidepressant in wt mice in our study (*Fig. 5.9 B*). We employed a tail suspension procedure that has been previously validated (Can *et al.*, 2012); nevertheless, unknown differences in variables between labs can affect success of behavioural tests, so future experiments will also include use of a known antidepressant, such as the tricyclic agent desipramine, as a positive control. Furthermore, antidepressant efficacy in the tail suspension test can vary widely with mouse strain (van der Heyden *et al.*, 1987) – perhaps the α2^{Q241M} mutation could be tried in an alternative background strain to C57BL/6J, in case there is some interaction of strain with antidepressant efficacy of THDOC. Unsurprisingly on the basis of results in other tests, THDOC increases immobility in hom mice in the tail suspension test, probably representing the ataxic effects of the drug (rather than an induction of behavioural despair).

Interestingly, although allopregnanolone levels are reduced in depressed individuals, THDOC levels are increased; antidepressant SSRIs increase allopregnanolone and decrease THDOC (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000). Furthermore, whilst

antidepressant effects of progesterone and allopregnanolone have been extensively verified in animal models (Khisti *et al.*, 2000; e.g. Hirani *et al.*, 2002; Shirayama *et al.*, 2011), we are unaware of any references demonstrating antidepression with THDOC. Given that both compounds are $GABA_A$ receptor potentiators, it is not clear why allopregnanolone could be antidepressant whilst THDOC is not, but perhaps there are subtle differences in their action *in vivo*, such as differential effects on non-$GABA_A$ receptor targets, that account for this discrepancy. Furthermore, the sulphated neurosteroids DHEA-sulphate and pregnenolone-sulphate, both negative allosteric modulators of $GABA_A$ receptors, also demonstrate antidepressant efficacy in the tail suspension test, probably via actions at sigma receptors (Dhir & Kulkarni, 2008). It would therefore seem that antidepressant action by neurosteroids is not restricted to $GABA_A$ receptor potentiation.

5.3.4. Exploring the mechanism underlying THDOC's ataxic effects in α2^{Q241M} mice

Several mechanisms could account for the reduced activity of THDOC-injected hom mice in the plus maze, light-dark box and tail suspension tests, including sedation/sleeping, freezing or impaired motor performance. Freezing is a normal rodent fear response; however, given that 20 mg/kg THDOC was activity-reducing but also anxiolytic in hom mice on the elevated plus maze, fear-induced freezing is unlikely to explain the reduced mobility. Sedation could be studied in several ways, including loss-of-righting reflex tests or by screening changes in electro-encephalogram (EEG) on THDOC injection; the latter may be more relevant to this work because the marked activity reductions are occurring at doses below the threshold for loss-of-righting reflex. During this project, THDOC's motor-impairing effects were assessed using an accelerating rotarod test (Jones & Roberts, 1968). The performances of untrained wt and hom mice were similar in this procedure (*Fig. 5.10 A*), suggesting the α2^{Q241M} mutation has no effect on motor function. After training, mice achieve a better

and more consistent performance on the rod; injection of vehicle or 10 mg/kg THDOC did not affect performance, whilst a similar degree of impairment was observed for both wt and hom animals after a 20 mg/kg dose (*Fig. 5.10 B*). We cannot rule out a genotypic difference at other doses of THDOC, but our data suggest that differences in motor impairment do not account for the differences in activity effects of THDOC.

There are some important differences between the rotarod test and the other tests described in this chapter: mice are habituated to handling during the course of their training (cf. other tests, where the mouse is only handled for routine husbandry and ear notching), and are effectively being forced to walk, in order to avoid falling off the rod (cf. other tests, where mice have a free choice over whether or not to move). It is difficult to say whether the different natures of these tests may affect sensitivity of test subjects to THDOC's ataxic effects, but we can at least be sure that there are no issues of tolerance because all mice are drug naïve. Even though different tests do appear to have slight differences in dose-responses (e.g. significant anxiolysis for wt mice is achieved at 10 mg/kg THDOC in the plus maze, but only at 20 mg/kg in the light-dark box), the activity effects in hom mice appear to be consistent across tests. If we therefore consider activity effects to be similarly dose-sensitive in the rotarod, then we have further support that motor impairment does not explain the activity effects: 10 mg/kg THDOC does not impair hom mice on the rotarod, but significantly reduces activity in the light-dark box and elevated plus maze.

To corroborate the above results we could employ alternative tests for motor impairment, such as the inverted screen test (Coughenour *et al.*, 1977), which has been argued a more sensitive test to motor impairing effects of drug. Perhaps a wider range of doses of THDOC should be employed on both tests, to ensure there are no genotypic differences in motor impairment at other drug concentrations.

A further explanation for activity effects is a hypersedative effect of THDOC in hom mice; several possible mechanisms could account for increased sedation. If, for example, the α1-type $GABA_A$ receptors are involved in sedative effects of neurosteroids, as they are for benzodiazepines (Rudolph *et al.*, 1999), increased sedation could result if there is an increased activity of these subunits in hom α2^{Q241M} knock-ins. We can rule out a change in the α1 subunit at the level of protein expression with our Western blot and immunofluorescence work, but these approaches give no indication of the activity of these ion channels. The faster decay kinetics for control sIPSCs recorded from CA1 pyramidal cells, dentate gyrus granule cells and nucleus accumbens medium spiny neurons (*Table 4.1*) may well be consistent with an increased contribution of α1-type $GABA_A$ receptors to these sIPSCs (an increased proportion of α1 vs. α2 subunits has been shown to reduce decay time of mIPSCs and evoked IPSCs (Okada *et al.*, 2000)). We also cannot rule out a mechanism whereby hom mice have a greater level of neurosteroids at baseline, such that after THDOC injection, brain neurosteroid levels may be sufficient to pass the sedative threshold in hom, but not wt mice. Additional experiments are needed to address the validity of these proposals.

Sedation, as measured by a reduced locomotor activity, is often taken as an indicator of hypnosis, but using EEG fingerprints, it is possible to dissociate hypnosis from sedation (Mohler, 2006a). Future experiments probing the mechanism underlying THDOC's hyper-sedative effects will therefore utilise EEG studies. If the mutation has led to an enhanced sensitivity to the hypnotic effects of neurosteroids, one may predict a reduced sleep latency following neurosteroid injection and increases in sleep vs. waking time. Potentiation of GABAergic transmission by diazepam has also been shown to have number of effects on rodent EEG patterns, including a rise in theta band (6-11 Hz) power during rapid eye movement (REM) sleep, decreased delta band (0.75-4 Hz) power in non-REM sleep and a reduced theta peak frequency during waking (Tobler *et al.*, 2001; Kopp *et al.*, 2004). These investigators found that the sleep-state EEG changes are attenuated in benzodiazepine-insensitive α2^{H101R} knock-in mice, but not in α1^{H101R} mice, suggesting hypnotic effects may relate to

benzodiazepine function at α2-, rather than α1-type $GABA_A$ receptors. It would therefore be interesting to perform similar experiments to probe the effects of $\alpha2^{Q241M}$ mutation on EEG patterns under basal conditions, and after injection of neurosteroids.

5.4. Conclusions

1. Endogenous neurosteroids present at baseline have tonic anxiolytic effects, but probably not antidepressant effects, via α2-type $GABA_A$ receptors.

2. Anxiolytic effects of systemically-applied neurosteroids are mediated, at least partly via α2-type $GABA_A$ receptors. Neurosteroids targeting the α2-type $GABA_A$ receptor may therefore represent an appropriate treatment strategy for anxiety disorders.

3. The $\alpha2^{Q241M}$ mutation does not abolish anxiolytic effects of other $GABA_A$ receptor potentiators, pentobarbital or diazepam. However, disrupted diazepam anxiolysis in the light-dark box suggest that neurosteroid responses on α2 subunits may also be required for benzodiazepine anxiolysis.

4. Systemically applied THDOC is not antidepressant in the tail-suspension test, and therefore may have distinct roles in anxiety and depression compared to the related neurosteroid allopregnanolone.

5. The ataxic effect of injected THDOC in α2^{Q241M} mutant mice does not derive from a hypersensitivity to motor impairing effects of this compound.

Chapter 6: General Discussion

6.1. Endogenous neurosteroids: functions at α2-type $GABA_A$ receptors

6.1.1. Anxiolysis but not anti-depression in unperturbed state

Previous work has implicated signalling through α2-type $GABA_A$ receptors as being an important component of anxiety circuitry and anxiolysis (Low *et al.*, 2000; Dixon *et al.*, 2008), and suggested that neurosteroids may act as endogenous anxiolytics (Crawley *et al.*, 1986; Purdy *et al.*, 1991; Wieland *et al.*, 1991; Barbaccia *et al.*, 1998). Our work provides the first direct demonstration of a link between the two: that the endogenous anxiolytic function of neurosteroids depends on potentiation of signalling through α2-type $GABA_A$ receptors. Lacking this function, homozygous $\alpha2^{Q241M}$ mice have a more anxious phenotype at baseline than their wild-type littermates.

Anxiety and depression are frequently comorbid, and so these disorders may have common underlying mechanisms (Hirschfeld, 2001; Nutt *et al.*, 2006). Indeed depression has also been associated with reduced GABAergic signalling (Luscher *et al.*, 2011b; Smith & Rudolph, 2012), possibly at α2-type $GABA_A$ receptors (Vollenweider *et al.*, 2011). Further, there are proposals that antidepressants achieve their effects by restoring a deficit in allopregnanolone levels (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000). One may therefore predict a depressed phenotype to be imparted by knocking-in the $\alpha2^{Q241M}$ mutation. However, we do not observe such a phenotype, using just one test, the tail suspension test. It is therefore possible that anxiety and depression are less tightly coupled than currently thought, with depression less closely linked with neurosteroid regulation of α2-type $GABA_A$ receptors.

6.1.2. α2^{Q241M} knock-in has no effect on receptor expression

Fluctuating levels of endogenous neurosteroids can influence the expression of $GABA_A$ receptor subunits. For example, concomitant with the allopregnanolone level rises in the estrus cycle of mice, hippocampal expression of δ subunits increases and that of γ2 subunits is decreased (Maguire *et al.*, 2005). However, we have found no change in expression of $GABA_A$ receptor α subunits in our male α2^{Q241M} knock-ins. This would suggest that basal neurosteroid potentiation at α2-subunits in male mice has little influence on the expression of $GABA_A$ receptor subunits.

6.1.3. Contribution of α2-type $GABA_A$ receptors to phasic and tonic transmission

On the basis of the effects of the α2^{Q241M} mutation in recombinant expression systems, one would predict that GABAergic neurotransmission will respond normally to benzodiazepines, but have an impaired response to neurosteroids (when currents are carried by α2-type $GABA_A$ receptors). Our recordings from three neuronal cell types appear entirely consistent with these predictions. Synaptic currents in wild-type animals respond to diazepam (500 nM) and THDOC (100 nM) with a similar prolongation of decay time (i.e. enhanced inhibitory neurotransmission). In homozygous α2^{Q241M} mice, however, only IPSC prolongation by diazepam is normal, and the response to THDOC is diminished (CA1 PCs) or ablated (NAcc MSNs and DG GCs). We therefore propose that α2-type $GABA_A$ receptors contribute significantly to the synaptic events in these cell types, and that the residual response of CA1 PCs to THDOC probably represents a ~50% contribution from the unaltered α subunit isoforms that are also expressed in these cells (α1, α3, α4 and α5). This result was to be expected given that α2 subunits play a role in synaptic targeting of $GABA_A$ receptors (Tretter *et al.*, 2008; Wu *et al.*, 2012) and are strongly

expressed in these cells (*Fig. 3.4*; Sperk *et al.*, 1997; Pirker *et al.*, 2000; Sieghart & Sperk, 2002; Mohler, 2006a).

The results we observe with tonic currents were perhaps less expected. THDOC increased the amplitude of the tonic currents recorded from DG GCs of wild-type animals; there was no such response in cells from homozygous $\alpha2^{Q241M}$ mice, indicating a requirement for potentiation at the α2 subunit. We believe this represents the first direct support for $GABA_A$ receptors containing the α2 subunit playing a role in tonic currents. This is particularly interesting given that the α2 subunit has been proposed to mostly assemble as α2β2/3γ2 receptors (McKernan & Whiting, 1996; Sieghart & Sperk, 2002; Mohler, 2006a), and the α2 subunit plays a role in directing receptors to synaptic, rather than extrasynaptic sites (Tretter *et al.*, 2008; Wu *et al.*, 2012). Such a dual role for α2-type $GABA_A$ receptors is not unreasonable; indeed, α5βnγ2 receptor combinations contribute to both synaptic and tonic currents in hippocampal pyramidal cells (Banks *et al.*, 1998; Caraiscos *et al.*, 2004; Prenosil *et al.*, 2006). Interestingly, however, the synaptic events – but not tonic currents – in our wild-type DG GC recordings show a response to diazepam. This difference in pharmacological profile would implicate α2βnγ2 receptors in the synaptic currents, and γ-subunit-lacking receptor combinations in the tonic currents (Pritchett *et al.*, 1989). We therefore propose that the THDOC-sensitive tonic current we observe in DG GCs may instead involve receptors of combination α2βn or α2βnδ.

6.2. The therapeutic potential of neurosteroids in anxiety and depression

6.2.1. THDOC is not antidepressant

Depression has been proposed to result from a deficiency in neurosteroids, because allopregnanolone levels are diminished in the plasma and CSF of depressed patients, and this deficit is ameliorated by treatment with a range of

antidepressants (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000). Indeed, selective-serotonin reuptake inhibitor (SSRI) antidepressants rapidly increase allopregnanolone production in rodent models (Uzunov *et al.*, 1996). Consistent with neurosteroids being the mediators of antidepression, antidepressant effects are evident with progesterone and allopregnanolone in animal models (e.g. Khisti *et al.*, 2000; Hirani *et al.*, 2002; Shirayama *et al.*, 2011). There are, however, a number of problems with this proposal:

1. Humans require chronic SSRI treatment before a benefit is observed, whilst the antidepressant effects are immediate in rodents (see discussion by Cryan *et al.*, 2005). Interestingly, chronic fluoxetine administration in rats actually decreases the plasma and brain concentrations of THDOC and allopregnanolone (Serra *et al.*, 2002).
2. Other non-SSRI antidepressants, such as imipramine, are clinically effective, but appear not to act by influencing neurosteroid metabolism (Uzunov *et al.*, 1996; Griffin & Mellon, 1999).
3. The changes in THDOC levels oppose those of allopregnanolone in depressed patients (in that THDOC levels increase in depression and are decreased by antidepressants (Romeo *et al.*, 1998; Uzunova *et al.*, 1998; Strohle *et al.*, 1999; Strohle *et al.*, 2000)).
4. We were unable to demonstrate an effect of THDOC in the tail suspension test – i.e. THDOC appears not to act as an antidepressant.

Points 3 and 4 are particularly troubling when considering the suggestions that the antidepressant function of neurosteroids occurs by enhancing GABAergic signalling (e.g. Khisti *et al.*, 2000). Both allopregnanolone and THDOC are positive allosteric modulators of $GABA_A$ receptors, so it is unclear why the increased THDOC in depressed patients would not have an antidepressant effect. Perhaps there are subtle differences between the action of allopregnanolone and THDOC *in vivo*, such as differential effects on non-$GABA_A$ receptor targets, allowing only the former to be antidepressant. Interestingly, sulphated steroids, which are negative allosteric modulators of $GABA_A$ receptors, are also antidepressant in the mouse tail-suspension test,

probably via actions at sigma receptors (Dhir & Kulkarni, 2008). Furthermore, benzodiazepines – classical potentiators of GABAergic transmission – are not generally antidepressant; only alprazolam has demonstrated any efficacy in depression (Laakmann *et al.*, 1996). The antidepressant action of neurosteroids is therefore incompletely understood, and is probably not restricted to $GABA_A$ receptor potentiation.

6.2.2. α2-type $GABA_A$ receptors are an appropriate target for neurosteroid anxiolytics

The anxiolytic response to THDOC injection in both elevated plus maze and light-dark box tests is disrupted by the $\alpha2^{Q241M}$ knock-in. These results are consistent with our hypothesis that α2-type $GABA_A$ receptors will be mediators of neurosteroid anxiolysis. We propose that, analogous to benzodiazepines, the sedative effects of neurosteroids will involve the α1 isoform. If this is the case, subunit selective neurosteroid analogues (with efficacy at α2 and no action at α1 subunits) would be non-sedative anxiolytics – opening an alternative avenue for drug design to the selective benzodiazepine approach.

6.3. Remaining questions and future work

6.3.1. Screening for compensatory changes in $\alpha2^{Q241M}$ mice

Our approach to screening for compensatory alterations in $\alpha2^{Q241M}$ mice was to focus on the total protein expression levels for $GABA_A$ subunits α1-α5, as likely candidates for compensatory changes. Whilst our Western blot and immunofluorescence data allow us to conclude that the phenotypes of this transgenic mouse are not a result of large changes in expression of $GABA_A$ receptors, they do not rule out changes in the activity of their ion channels.

Indeed, baseline sIPSCs in knock-in mice decay faster than those of wild-type littermates, reducing the charge transfer per synaptic event, which may indicate some compensatory response to the mutation. However, there are numerous observations that would argue against compensatory changes. Firstly, the amplitude and frequency of sIPSCs is unchanged, indicating no alteration in the number of $GABA_A$ receptors expressed at the synaptic site, and/or a lack of alteration in presynaptic GABA release. Secondly, the sIPSC decay time prolongation by 500 nM diazepam is undiminished by the $\alpha2^{Q241M}$ mutation in DG GCs and NAcc MSNs, further indicating an unaltered function of synaptic $GABA_A$ receptors. Finally, tonic current amplitudes are unchanged in knock-in mice, suggesting unchanged activity of extrasynaptic $GABA_A$ receptors. We propose that the difference in baseline sIPSC decay times can instead be explained by a loss of sensitivity to endogenous neurosteroids within the brain slice. Indeed, other investigators have supported a role for endogenous neurosteroids in modulating baseline IPSC decay times in brain slice recordings (Puia *et al.*, 2003).

It would be surprising if loss of neurosteroid function at α2-type $GABA_A$ receptors does not induce some sort of compensatory change in the neuronal network, especially since the basal inhibitory synaptic tone has been reduced in the knock-in mice. We therefore propose that future work should use a microarray or proteomic approach to screen for differences in expression patterns in $\alpha2^{Q241M}$ vs. wild-type mice. Furthermore, other avenues for compensation should be considered, particularly screening for alterations in neurosteroid levels. Since baseline inhibitory GABAergic neurotransmission in the hippocampus is defective in the $\alpha2^{Q241M}$ mutant mice, one may predict a heightened activity of the HPA axis in these animals (see *Section 1.3.1*). If this is the case, there would also be elevated production of neurosteroids and other steroids in the adrenal cortex. Indeed the heightened plasma and CSF levels of THDOC found in depressed patients may be a consequence of the HPA axis hyperactivity in these individuals (Shen *et al.*, 2010). Future work will therefore compare the brain neurosteroid profile of $\alpha2^{Q241M}$ mice with their wild-type counterparts.

6.3.2. Understanding the activity effects

Several mechanisms could account for the reduced locomotor activity observed in knock-in mice after THDOC injection, including sedation/sleeping, freezing or impaired motor performance. Our rotarod data suggest that $\alpha2^{Q241M}$ mice are not hyper-sensitive to the motor-impairing effects of THDOC. Future experiments probing the mechanism underlying the hyper-sedative effects will utilise EEG studies.

6.3.3. Future work with $\alpha2^{Q241M}$ mutant mice

Would neurosteroid therapy be addictive?

The long-term use of benzodiazepines is precluded by the development of tolerance and addiction (File, 1985; O'Brien, 2005). Would a neurosteroid-based therapy suffer the same problems? Some investigators found that no tolerance developed to the anticonvulsant effects of pregnenolone or the neurosteroid analogue, ganaxolone, in rodents (Kokate *et al.*, 1998; Reddy & Rogawski, 2000), and ganaxolone has been used in some epileptic patients for a number of years (Nohria & Giller, 2007). However, other investigators have argued that tolerance does develop in response to chronic rises in neurosteroid levels (see review by Turkmen *et al.*, 2011). Furthermore, disorders such as catamenial epilepsy, post-partum depression and premenstrual dysphoric disorder have all been suggested to represent a withdrawal from high endogenous neurosteroid levels (Smith *et al.*, 1998; Bloch *et al.*, 2000; Beckley & Finn, 2007; Turkmen *et al.*, 2011), which would suggest that withdrawal would develop if neurosteroid therapies were abruptly terminated.

The above observations do not necessarily suggest that neurosteroid therapies would be addictive, and a key question is whether they would have rewarding

properties. The mechanisms underlying benzodiazepine reward are a subject of debate. Encouragingly, there have been suggestions that α1-type $GABA_A$ receptors in the ventral tegmental area are key to benzodiazepine addiction, and that α2-selective compounds will have low addiction liability (Tan *et al.*, 2010; Tan *et al.*, 2011). If neurosteroids act in a similar way, then α2-targeting neurosteroids may prove to be non-addictive anxiolytics. However, α2 and α3-type $GABA_A$ receptors have recently been implicated in mediating some of the rewarding effects of benzodiazepines (which reduce the threshold for intracranial self-stimulation (ICSS) in wild-type but not $\alpha2^{H101R}$ or $\alpha3^{H126R}$ (benzodiazepine-insensitive knock-in) mice (Reynolds *et al.*, 2012)). More work is therefore required to further elucidate the roles of GABAergic signalling in the mesolimbic dopamine pathway in addiction. It would be interesting to probe whether neurosteroids have similar rewarding effects in an ICSS test, and whether this is altered in $\alpha2^{Q241M}$ mice.

Neurosteroids in relief of chronic pain?

Whilst many mechanisms underlie chronic pain (see review by Zeilhofer, 2008), a common emerging theme involves reduced inhibitory neurotransmission in the spinal cord dorsal horn. Potentiating inhibition via α2 subunit-containing $GABA_A$ receptors may be an appropriate therapeutic strategy for chronic pain syndromes (Jasmin *et al.*, 2003; Zeilhofer *et al.*, 2012). Furthermore, in models of hyperalgesia, endogenously-synthesised and applied neurosteroids are analgesic (Winter *et al.*, 2003; Poisbeau *et al.*, 2005). Using our $\alpha2^{Q241M}$ mice, we can probe whether signalling through α2-type $GABA_A$ receptors by neurosteroids plays a role in pain physiology, and determine if α2-selective neurosteroids would form an appropriate treatment strategy for neuropathic or inflammatory pain.

6.4. Concluding statement

Neurosteroids, such as allopregnanolone and THDOC, are important endogenous modulators of $GABA_A$ receptors. They are involved in numerous physiological processes, and are linked to several central nervous system disorders, including depression and anxiety. Their effects in animal models suggest they could be useful therapeutic agents, for example in anxiety, stress and mood disorders. We have used $\alpha2^{Q241M}$ knock-in mice to demonstrate that neurosteroid-mediated anxiolysis occurs via potentiation at α2 subunit-containing receptors. This not only informs us as to one of the key physiological functions of endogenous neurosteroids, but also identifies the α2 isoform as an appropriate target for generating receptor subtype-selective neurosteroid therapeutics for anxiety disorders.

Using our knock-in strain, we have also revealed new information with regards to the normal physiological roles of neurosteroids and $GABA_A$ receptors. We propose that neurosteroid potentiation at α2-subunits has little influence on the expression of $GABA_A$ receptors in male mice. Our electrophysiological characterization demonstrated a role for α2-type $GABA_A$ receptors not only in synaptic transmission, but also in mediating tonic currents in the dentate gyrus.

References

Akk G, Bracamontes J & Steinbach JH. (2001). Pregnenolone sulfate block of $GABA_A$ receptors: mechanism and involvement of a residue in the M2 region of the α subunit. *J Physiol* **532,** 673-684.

Amato A, Connolly CN, Moss SJ & Smart TG. (1999). Modulation of neuronal and recombinant $GABA_A$ receptors by redox reagents. *J Physiol* **517 (Pt 1),** 35-50.

Arancibia-Carcamo IL & Kittler JT. (2009). Regulation of $GABA_A$ receptor membrane trafficking and synaptic localization. *Pharmacol Ther* **123,** 17-31.

Arancibia-Carcamo IL, Yuen EY, Muir J, Lumb MJ, Michels G, Saliba RS, Smart TG, Yan Z, Kittler JT & Moss SJ. (2009). Ubiquitin-dependent lysosomal targeting of $GABA_A$ receptors regulates neuronal inhibition. *Proc Natl Acad Sci U S A* **106,** 17552-17557.

Atack JR. (2008). $GABA_A$ receptor subtype-selective efficacy: TPA023, an $\alpha2/\alpha3$ selective non-sedating anxiolytic and $\alpha5$IA, an $\alpha5$ selective cognition enhancer. *CNS Neurosci Ther* **14,** 25-35.

Atack JR. (2010). $GABA_A$ receptor $\alpha2/\alpha3$ subtype-selective modulators as potential nonsedating anxiolytics. *Curr Top Behav Neurosci* **2,** 331-360.

Atack JR, Hutson PH, Collinson N, Marshall G, Bentley G, Moyes C, Cook SM, Collins I, Wafford K, McKernan RM & Dawson GR. (2005). Anxiogenic properties of an inverse agonist selective for $\alpha3$ subunit-containing $GABA_A$ receptors. *Br J Pharmacol* **144,** 357-366.

Atack JR, Wafford KA, Street LJ, Dawson GR, Tye S, Van Laere K, Bormans G, Sanabria-Bohorquez SM, De Lepeleire I, de Hoon JN, Van Hecken A, Burns HD, McKernan RM, Murphy MG & Hargreaves RJ. (2011). MRK-409 (MK-0343), a $GABA_A$ receptor subtype-selective partial agonist, is a non-sedating anxiolytic in preclinical species but causes sedation in humans. *J Psychopharmacol* **25,** 314-328.

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF & Orser BA. (2001). Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. *Mol Pharmacol* **59,** 814-824.

Bailey DW. (1982). How pure are inbred strains of mice. *Immunology Today* **3,** 210-214.

Banks MI, Li TB & Pearce RA. (1998). The synaptic basis of $GABA_{A,slow}$. *J Neurosci* **18,** 1305-1317.

Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, Zhang WN, Pothuizen HH & Feldon J. (2004). Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev* **28,** 273-283.

Barbaccia ML, Colombo G, Affricano D, Carai MA, Vacca G, Melis S, Purdy RH & Gessa GL. (2002). $GABA_B$ receptor-mediated increase of neurosteroids by γ-hydroxybutyric acid. *Neuropharmacology* **42,** 782-791.

Barbaccia ML, Concas A, Serra M & Biggio G. (1998). Stress and neurosteroids in adult and aged rats. *Exp Gerontol* **33,** 697-712.

Barbaccia ML, Roscetti G, Trabucchi M, Purdy RH, Mostallino MC, Perra C, Concas A & Biggio G. (1996). Isoniazid-induced inhibition of GABAergic transmission enhances neurosteroid content in the rat brain. *Neuropharmacology* **35,** 1299-1305.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN & Langer SZ. (1998). International Union of Pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* **50,** 291-313.

Beckley EH & Finn DA. (2007). Inhibition of progesterone metabolism mimics the effect of progesterone withdrawal on forced swim test immobility. *Pharmacol Biochem Behav* **87,** 412-419.

Beckley EH, Scibelli AC & Finn DA. (2011). Progesterone receptor antagonist CDB-4124 increases depression-like behavior in mice without affecting locomotor ability. *Psychoneuroendocrinology* **36,** 824-833.

Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG & Moss SJ. (2001). $GABA_A$ receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. *Nat Neurosci* **4,** 908-916.

Belelli D, Casula A, Ling A & Lambert JJ. (2002). The influence of subunit composition on the interaction of neurosteroids with $GABA_A$ receptors. *Neuropharmacology* **43,** 651-661.

Belelli D & Herd MB. (2003). The contraceptive agent Provera enhances $GABA_A$ receptor-mediated inhibitory neurotransmission in the rat hippocampus: evidence for endogenous neurosteroids? *J Neurosci* **23,** 10013-10020.

Belelli D, Herd MB, Mitchell EA, Peden DR, Vardy AW, Gentet L & Lambert JJ. (2006). Neuroactive steroids and inhibitory neurotransmission: Mechanisms of action and physiological relevance. *Neuroscience* **138,** 821-829.

Ben-Ari Y. (2002). Excitatory actions of GABA during development: the nature of the nurture. *Nat Rev Neurosci* **3,** 728-739.

Benjamini Y, Drai D, Elmer G, Kafkafi N & Golani I. (2001). Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* **125,** 279-284.

Benke D, Fritschy JM, Trzeciak A, Bannwarth W & Mohler H. (1994). Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. *J Biol Chem* **269,** 27100-27107.

Benke D, Mertens S, Trzeciak A, Gillessen D & Mohler H. (1991). $GABA_A$ receptors display association of γ2-subunit with α1- and β2/3-subunits. *J Biol Chem* **266,** 4478-4483.

Benson JA, Low K, Keist R, Mohler H & Rudolph U. (1998). Pharmacology of recombinant γ-aminobutyric $acid_A$ receptors rendered diazepam-insensitive by point-mutated α subunits. *FEBS Lett* **431,** 400-404.

Bernardi F, Salvestroni C, Casarosa E, Nappi RE, Lanzone A, Luisi S, Purdy RH, Petraglia F & Genazzani AR. (1998). Aging is associated with changes in allopregnanolone concentrations in brain, endocrine glands and serum in male rats. *Eur J Endocrinol* **138,** 316-321.

Bianchi L, Ballini C, Colivicchi MA, Della Corte L, Giovannini MG & Pepeu G. (2003). Investigation on acetylcholine, aspartate, glutamate and GABA extracellular levels from ventral hippocampus during repeated exploratory activity in the rat. *Neurochem Res* **28,** 565-573.

Bianchi MT & Macdonald RL. (2003). Neurosteroids shift partial agonist activation of $GABA_A$ receptor channels from low- to high-efficacy gating patterns. *J Neurosci* **23,** 10934-10943.

Biedermann K & Schoch P. (1995). Do neuroactive steroids cause fatigue in pregnancy? *Eur J Obstet Gynecol Reprod Biol* **58,** 15-18.

Bixo M, Andersson A, Winblad B, Purdy RH & Backstrom T. (1997). Progesterone, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnane-20-one in specific regions

of the human female brain in different endocrine states. *Brain Res* **764,** 173-178.

Blanchard DC, Blanchard RJ, Tom P & Rodgers RJ. (1990). Diazepam changes risk assessment in an anxiety/defense test battery. *Psychopharmacology (Berl)* **101,** 511-518.

Bloch M, Schmidt PJ, Danaceau M, Murphy J, Nieman L & Rubinow DR. (2000). Effects of gonadal steroids in women with a history of postpartum depression. *Am J Psychiatry* **157,** 924-930.

Blumstein LK & Crawley JN. (1983). Further characterization of a simple, automated exploratory model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav* **18,** 37-40.

Bracamontes JR & Steinbach JH. (2009). Steroid interaction with a single potentiating site is sufficient to modulate GABA-A receptor function. *Mol Pharmacol* **75,** 973-981.

Brandon N, Jovanovic J & Moss S. (2002). Multiple roles of protein kinases in the modulation of γ-aminobutyric acid$_A$ receptor function and cell surface expression. *Pharmacol Ther* **94,** 113-122.

Brickley SG, Cull-Candy SG & Farrant M. (1999). Single-channel properties of synaptic and extrasynaptic GABA$_A$ receptors suggest differential targeting of receptor subtypes. *J Neurosci* **19,** 2960-2973.

Brickley SG, Revilla V, Cull-Candy SG, Wisden W & Farrant M. (2001). Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. *Nature* **409,** 88-92.

Brown ES, Rush AJ & McEwen BS. (1999). Hippocampal remodeling and damage by corticosteroids: implications for mood disorders. *Neuropsychopharmacology* **21,** 474-484.

Brown N, Kerby J, Bonnert TP, Whiting PJ & Wafford KA. (2002). Pharmacological characterization of a novel cell line expressing human $\alpha 4\beta 3\delta$ GABA$_A$ receptors. *Br J Pharmacol* **136,** 965-974.

Brussaard AB, Kits KS, Baker RE, Willems WP, Leyting-Vermeulen JW, Voorn P, Smit AB, Bicknell RJ & Herbison AE. (1997). Plasticity in fast synaptic inhibition of adult oxytocin neurons caused by switch in GABA$_A$ receptor subunit expression. *Neuron* **19,** 1103-1114.

Brussaard AB, Wossink J, Lodder JC & Kits KS. (2000). Progesterone-metabolite prevents protein kinase C-dependent modulation of γ-aminobutyric acid type A receptors in oxytocin neurons. *Proc Natl Acad Sci U S A* **97,** 3625-3630.

Can A, Dao DT, Terrillion CE, Piantadosi SC, Bhat S & Gould TD. (2012). The tail suspension test. *J Vis Exp***,** e3769.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF & Orser BA. (2004). Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing γ-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* **101,** 3662-3667.

Carter RB, Wood PL, Wieland S, Hawkinson JE, Belelli D, Lambert JJ, White HS, Wolf HH, Mirsadeghi S, Tahir SH, Bolger MB, Lan NC & Gee KW. (1997). Characterization of the anticonvulsant properties of ganaxolone (CCD 1042; 3α-hydroxy-3β-methyl-5α-pregnan-20-one), a selective, high-affinity, steroid modulator of the γ-aminobutyric acid$_A$ receptor. *J Pharmacol Exp Ther* **280,** 1284-1295.

Chandra D, Korpi ER, Miralles CP, De Blas AL & Homanics GE. (2005). GABA$_A$ receptor γ2 subunit knockdown mice have enhanced anxiety-like behavior but unaltered hypnotic response to benzodiazepines. *BMC Neurosci* **6,** 30.

Chang Y, Wang R, Barot S & Weiss DS. (1996). Stoichiometry of a recombinant GABA$_A$ receptor. *J Neurosci* **16,** 5415-5424.

Chiang PH, Wu PY, Kuo TW, Liu YC, Chan CF, Chien TC, Cheng JK, Huang YY, Chiu CD & Lien CC. (2012). GABA is depolarizing in hippocampal dentate granule cells of the adolescent and adult rats. *J Neurosci* **32,** 62-67.

Chisari M, Eisenman LN, Covey DF, Mennerick S & Zorumski CF. (2010). The sticky issue of neurosteroids and GABA$_A$ receptors. *Trends Neurosci* **33,** 299-306.

Concas A, Mostallino MC, Porcu P, Follesa P, Barbaccia ML, Trabucchi M, Purdy RH, Grisenti P & Biggio G. (1998). Role of brain allopregnanolone in the plasticity of γ-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. *Proc Natl Acad Sci U S A* **95,** 13284-13289.

Connolly CN, Kittler JT, Thomas P, Uren JM, Brandon NJ, Smart TG & Moss SJ. (1999). Cell surface stability of γ-aminobutyric acid type A receptors. Dependence on protein kinase C activity and subunit composition. *J Biol Chem* **274,** 36565-36572.

Cooper EJ, Johnston GA & Edwards FA. (1999). Effects of a naturally occurring neurosteroid on $GABA_A$ IPSCs during development in rat hippocampal or cerebellar slices. *J Physiol* **521 Pt 2,** 437-449.

Corpechot C, Collins BE, Carey MP, Tsouros A, Robel P & Fry JP. (1997). Brain neurosteroids during the mouse oestrous cycle. *Brain Res* **766,** 276-280.

Corringer PJ, Poitevin F, Prevost MS, Sauguet L, Delarue M & Changeux JP. (2012). Structure and pharmacology of pentameric receptor channels: from bacteria to brain. *Structure* **20,** 941-956.

Coughenour LL, McLean JR & Parker RB. (1977). A new device for the rapid measurement of impaired motor function in mice. *Pharmacol Biochem Behav* **6,** 351-353.

Crawley JN, Glowa JR, Majewska MD & Paul SM. (1986). Anxiolytic activity of an endogenous adrenal steroid. *Brain Res* **398,** 382-385.

Crawley JN & Goodwin FK. (1980). Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol Biochem Behav* **13,** 167-170.

Crestani F, Lorez M, Baer K, Essrich C, Benke D, Laurent JP, Belzung C, Fritschy JM, Luscher B & Mohler H. (1999). Decreased $GABA_A$-receptor clustering results in enhanced anxiety and a bias for threat cues. *Nat Neurosci* **2,** 833-839.

Crestani F, Low K, Keist R, Mandelli M, Mohler H & Rudolph U. (2001). Molecular targets for the myorelaxant action of diazepam. *Mol Pharmacol* **59,** 442-445.

Cryan JF, Mombereau C & Vassout A. (2005). The tail suspension test as a model for assessing antidepressant activity: Review of pharmacological and genetic studies in mice. *Neurosci Biobehav Rev* **29,** 571-625.

de Lanerolle NC, Kim JH, Williamson A, Spencer SS, Zaveri HP, Eid T & Spencer DD. (2003). A retrospective analysis of hippocampal pathology in human temporal lobe epilepsy: evidence for distinctive patient subcategories. *Epilepsia* **44,** 677-687.

Dean B, Scarr E & McLeod M. (2005). Changes in hippocampal $GABA_A$ receptor subunit composition in bipolar 1 disorder. *Brain Res Mol Brain Res* **138,** 145-155.

Dhir A & Kulkarni S. (2008). Involvement of sigma (σ1) receptors in modulating the anti-depressant effect of neurosteroids (dehydroepiandrosterone or pregnenolone) in mouse tail-suspension test. *J Psychopharmacol* **22,** 691-696.

Dhir A & Rogawski MA. (2012). Role of neurosteroids in the anticonvulsant activity of midazolam. *Br J Pharmacol* **165,** 2684-2691.

Dias R, Sheppard WF, Fradley RL, Garrett EM, Stanley JL, Tye SJ, Goodacre S, Lincoln RJ, Cook SM, Conley R, Hallett D, Humphries AC, Thompson SA, Wafford KA, Street LJ, Castro JL, Whiting PJ, Rosahl TW, Atack JR, McKernan RM, Dawson GR & Reynolds DS. (2005). Evidence for a significant role of α3-containing $GABA_A$ receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci* **25,** 10682-10688.

Dixon CI, Morris HV, Breen G, Desrivieres S, Jugurnauth S, Steiner RC, Vallada H, Guindalini C, Laranjeira R, Messas G, Rosahl TW, Atack JR, Peden DR, Belelli D, Lambert JJ, King SL, Schumann G & Stephens DN. (2010). Cocaine effects on mouse incentive-learning and human addiction are linked to α2 subunit-containing $GABA_A$ receptors. *Proc Natl Acad Sci U S A* **107,** 2289-2294.

Dixon CI, Rosahl TW & Stephens DN. (2008). Targeted deletion of the *GABRA2* gene encoding α2-subunits of $GABA_A$ receptors facilitates performance of a conditioned emotional response, and abolishes anxiolytic effects of benzodiazepines and barbiturates. *Pharmacol Biochem Behav* **90,** 1-8.

Dulawa SC & Hen R. (2005). Recent advances in animal models of chronic antidepressant effects: The novelty-induced hypophagia test. *Neurosci Biobehav Rev* **29,** 771-783.

Dunham NW & Miya TS. (1957). A note on a simple apparatus for detecting neurological deficit in rats and mice. *J Am Pharm Assoc Am Pharm Assoc (Baltim)* **46,** 208-209.

Earnheart JC, Schweizer C, Crestani F, Iwasato T, Itohara S, Mohler H & Luscher B. (2007). GABAergic control of adult hippocampal neurogenesis in relation to behavior indicative of trait anxiety and depression states. *J Neurosci* **27,** 3845-3854.

Essrich C, Lorez M, Benson JA, Fritschy JM & Luscher B. (1998). Postsynaptic clustering of major $GABA_A$ receptor subtypes requires the γ2 subunit and gephyrin. *Nat Neurosci* **1,** 563-571.

Fancsik A, Linn DM & Tasker JG. (2000). Neurosteroid modulation of GABA IPSCs is phosphorylation dependent. *J Neurosci* **20,** 3067-3075.

Fanselow MS & Dong HW. (2010). Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron* **65,** 7-19.

Farisello P, Boido D, Nieus T, Medrihan L, Cesca F, Valtorta F, Baldelli P & Benfenati F. (2012). Synaptic and extrasynaptic origin of the excitation/inhibition imbalance in the hippocampus of synapsin I/II/III knockout mice. *Cereb Cortex*.

Farrant M & Nusser Z. (2005). Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nat Rev Neurosci* **6,** 215-229.

Farrar SJ, Whiting PJ, Bonnert TP & McKernan RM. (1999). Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. *J Biol Chem* **274,** 10100-10104.

File SE. (1985). Tolerance to the behavioral actions of benzodiazepines. *Neurosci Biobehav Rev* **9,** 113-121.

Fischer F, Kneussel M, Tintrup H, Haverkamp S, Rauen T, Betz H & Wassle H. (2000). Reduced synaptic clustering of GABA and glycine receptors in the retina of the gephyrin null mutant mouse. *J Comp Neurol* **427,** 634-648.

Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T & Mohler H. (1992). Five subtypes of type A γ-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. *Proc Natl Acad Sci U S A* **89,** 6726-6730.

Fritschy JM & Brunig I. (2003). Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. *Pharmacol Ther* **98,** 299-323.

Glencorse TA, Bateson AN & Darlison MG. (1992). Differential Localization of Two Alternatively Spliced $GABA_A$ Receptor γ2-Subunit mRNAs in the Chick Brain. *Eur J Neurosci* **4,** 271-277.

Glykys J, Mann EO & Mody I. (2008). Which $GABA_A$ receptor subunits are necessary for tonic inhibition in the hippocampus? *J Neurosci* **28,** 1421-1426.

Glykys J & Mody I. (2007). The main source of ambient GABA responsible for tonic inhibition in the mouse hippocampus. *J Physiol* **582,** 1163-1178.

Griffin LD, Gong W, Verot L & Mellon SH. (2004). Niemann-Pick type C disease involves disrupted neurosteroidogenesis and responds to allopregnanolone. *Nat Med* **10,** 704-711.

Griffin LD & Mellon SH. (1999). Selective serotonin reuptake inhibitors directly alter activity of neurosteroidogenic enzymes. *Proc Natl Acad Sci U S A* **96,** 13512-13517.

Gunther U, Benson J, Benke D, Fritschy JM, Reyes G, Knoflach F, Crestani F, Aguzzi A, Arigoni M, Lang Y & et al. (1995). Benzodiazepine-insensitive mice

generated by targeted disruption of the γ2 subunit gene of γ-aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* **92,** 7749-7753.

Harney SC, Frenguelli BG & Lambert JJ. (2003). Phosphorylation influences neurosteroid modulation of synaptic $GABA_A$ receptors in rat CA1 and dentate gyrus neurones. *Neuropharmacology* **45,** 873-883.

Harris RA, Osterndorff-Kahanek E, Ponomarev I, Homanics GE & Blednov YA. (2011). Testing the silence of mutations: Transcriptomic and behavioral studies of $GABA_A$ receptor α1 and α2 subunit knock-in mice. *Neurosci Lett* **488,** 31-35.

Harrison NL & Simmonds MA. (1984). Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res* **323,** 287-292.

Harrison NL, Vicini S & Barker JL. (1987). A steroid anesthetic prolongs inhibitory postsynaptic currents in cultured rat hippocampal neurons. *J Neurosci* **7,** 604-609.

Hascoet M & Bourin M. (1998). A new approach to the light/dark test procedure in mice. *Pharmacol Biochem Behav* **60,** 645-653.

Hasler G, van der Veen JW, Tumonis T, Meyers N, Shen J & Drevets WC. (2007). Reduced prefrontal glutamate/glutamine and γ-aminobutyric acid levels in major depression determined using proton magnetic resonance spectroscopy. *Arch Gen Psychiatry* **64,** 193-200.

Heimer L, Alheid GF, de Olmos JS, Groenewegen HJ, Haber SN, Harlan RE & Zahm DS. (1997). The accumbens: beyond the core-shell dichotomy. *J Neuropsychiatry Clin Neurosci* **9,** 354-381.

Helms CM, Rossi DJ & Grant KA. (2012). Neurosteroid influences on sensitivity to ethanol. *Front Endocrinol (Lausanne)* **3,** 10.

Henkel V, Baghai TC, Eser D, Zill P, Mergl R, Zwanzger P, Schule C, Bottlender R, Jager M, Rupprecht R, Hegerl U, Moller HJ & Bondy B. (2004). The gamma amino butyric acid (GABA) receptor alpha-3 subunit gene polymorphism in unipolar depressive disorder: a genetic association study. *Am J Med Genet B Neuropsychiatr Genet* **126B,** 82-87.

Hibbs RE & Gouaux E. (2011). Principles of activation and permeation in an anion-selective Cys-loop receptor. *Nature* **474,** 54-60.

Hirani K, Khisti RT & Chopde CT. (2002). Behavioral action of ethanol in Porsolt's forced swim test: modulation by 3alpha-hydroxy-5alpha-pregnan-20-one. *Neuropharmacology* **43,** 1339-1350.

Hirschfeld RM. (2001). The comorbidity of major depression and anxiety disorders: Recognition and management in primary care. *Prim Care Companion J Clin Psychiatry* **3,** 244-254.

Hogg S. (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. *Pharmacol Biochem Behav* **54,** 21-30.

Honig A, Bartlett JR, Bouras N & Bridges PK. (1988). Amino acid levels in depression: a preliminary investigation. *J Psychiatr Res* **22,** 159-164.

Horiuchi Y, Nakayama J, Ishiguro H, Ohtsuki T, Detera-Wadleigh SD, Toyota T, Yamada K, Nankai M, Shibuya H, Yoshikawa T & Arinami T. (2004). Possible association between a haplotype of the GABA-A receptor alpha 1 subunit gene (GABRA1) and mood disorders. *Biol Psychiatry* **55,** 40-45.

Hosie AM, Clarke L, da Silva H & Smart TG. (2009). Conserved site for neurosteroid modulation of $GABA_A$ receptors. *Neuropharmacology* **56,** 149-154.

Hosie AM, Dunne EL, Harvey RJ & Smart TG. (2003). Zinc-mediated inhibition of $GABA_A$ receptors: discrete binding sites underlie subtype specificity. *Nat Neurosci* **6,** 362-369.

Hosie AM, Wilkins ME, da Silva HM & Smart TG. (2006). Endogenous neurosteroids regulate $GABA_A$ receptors through two discrete transmembrane sites. *Nature* **444,** 486-489.

Hosie AM, Wilkins ME & Smart TG. (2007). Neurosteroid binding sites on $GABA_A$ receptors. *Pharmacol Ther* **116,** 7-19.

Houston CM, Bright DP, Sivilotti LG, Beato M & Smart TG. (2009a). Intracellular chloride ions regulate the time course of GABA-mediated inhibitory synaptic transmission. *J Neurosci* **29,** 10416-10423.

Houston CM, He Q & Smart TG. (2009b). CaMKII phosphorylation of the $GABA_A$ receptor: receptor subtype- and synapse-specific modulation. *J Physiol* **587,** 2115-2125.

Hu W, Zhang MY, Czeh B, Flugge G & Zhang WQ. (2010). Stress impairs GABAergic network function in the hippocampus by activating nongenomic glucocorticoid receptors and affecting the integrity of the parvalbumin-expressing neuronal network. *Neuropsychopharmacology* **35,** 1693-1707.

Huang RQ & Dillon GH. (1999). Effect of extracellular pH on GABA-activated current in rat recombinant receptors and thin hypothalamic slices. *J Neurophysiol* **82,** 1233-1243.

Hyman SE & Malenka RC. (2001). Addiction and the brain: the neurobiology of compulsion and its persistence. *Nat Rev Neurosci* **2,** 695-703.

Ichikawa S, Sawada T, Nakamura Y & Morioka H. (1974). Ovarian secretion of pregnane compounds during the estrous cycle and pregnancy in rats. *Endocrinology* **94,** 1615-1620.

Item C & Sieghart W. (1994). Binding of γ-aminobutyric $acid_A$ receptors to tubulin. *J Neurochem* **63,** 1119-1125.

Jasmin L, Rabkin SD, Granato A, Boudah A & Ohara PT. (2003). Analgesia and hyperalgesia from GABA-mediated modulation of the cerebral cortex. *Nature* **424,** 316-320.

Jones BJ & Roberts DJ. (1968). A rotarod suitable for quantitative measurements of motor incoordination in naive mice. *Naunyn Schmiedebergs Arch Exp Pathol Pharmakol* **259,** 211.

Jovanovic JN, Thomas P, Kittler JT, Smart TG & Moss SJ. (2004). Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating $GABA_A$ receptor phosphorylation, activity, and cell-surface stability. *J Neurosci* **24,** 522-530.

Kanematsu T, Yasunaga A, Mizoguchi Y, Kuratani A, Kittler JT, Jovanovic JN, Takenaka K, Nakayama KI, Fukami K, Takenawa T, Moss SJ, Nabekura J & Hirata M. (2006). Modulation of $GABA_A$ receptor phosphorylation and membrane trafficking by phospholipase C-related inactive protein/protein phosphatase 1 and 2A signaling complex underlying brain-derived neurotrophic factor-dependent regulation of GABAergic inhibition. *J Biol Chem* **281,** 22180-22189.

Khisti RT, Chopde CT & Jain SP. (2000). Antidepressant-like effect of the neurosteroid 3α-hydroxy-5α-pregnan-20-one in mice forced swim test. *Pharmacol Biochem Behav* **67,** 137-143.

Khisti RT, Penland SN, VanDoren MJ, Grobin AC & Morrow AL. (2002). GABAergic neurosteroid modulation of ethanol actions. *World J Biol Psychiatry* **3,** 87-95.

Kittler JT, Chen G, Honing S, Bogdanov Y, McAinsh K, Arancibia-Carcamo IL, Jovanovic JN, Pangalos MN, Haucke V, Yan Z & Moss SJ. (2005). Phospho-dependent binding of the clathrin AP2 adaptor complex to $GABA_A$ receptors regulates the efficacy of inhibitory synaptic transmission. *Proc Natl Acad Sci U S A* **102,** 14871-14876.

Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG & Moss SJ. (2000). Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. *J Neurosci* **20,** 7972-7977.

Kittler JT & Moss SJ. (2001). Neurotransmitter receptor trafficking and the regulation of synaptic strength. *Traffic* **2,** 437-448.

Kittler JT & Moss SJ. (2003). Modulation of $GABA_A$ receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. *Curr Opin Neurobiol* **13,** 341-347.

Kittler JT, Rostaing P, Schiavo G, Fritschy JM, Olsen R, Triller A & Moss SJ. (2001). The subcellular distribution of GABARAP and its ability to interact with NSF suggest a role for this protein in the intracellular transport of $GABA_A$ receptors. *Mol Cell Neurosci* **18,** 13-25.

Kneussel M, Haverkamp S, Fuhrmann JC, Wang H, Wassle H, Olsen RW & Betz H. (2000). The γ-aminobutyric acid type A receptor ($GABA_AR$)-associated protein GABARAP interacts with gephyrin but is not involved in receptor anchoring at the synapse. *Proc Natl Acad Sci U S A* **97,** 8594-8599.

Knuesel I, Mastrocola M, Zuellig RA, Bornhauser B, Schaub MC & Fritschy JM. (1999). Short communication: altered synaptic clustering of $GABA_A$ receptors in mice lacking dystrophin (mdx mice). *Eur J Neurosci* **11,** 4457-4462.

Kofuji P, Wang JB, Moss SJ, Huganir RL & Burt DR. (1991). Generation of two forms of the γ-aminobutyric $acid_A$ receptor γ2-subunit in mice by alternative splicing. *J Neurochem* **56,** 713-715.

Kokate TG, Yamaguchi S, Pannell LK, Rajamani U, Carroll DM, Grossman AB & Rogawski MA. (1998). Lack of anticonvulsant tolerance to the neuroactive steroid pregnanolone in mice. *J Pharmacol Exp Ther* **287,** 553-558.

Koksma JJ, van Kesteren RE, Rosahl TW, Zwart R, Smit AB, Luddens H & Brussaard AB. (2003). Oxytocin regulates neurosteroid modulation of $GABA_A$ receptors in supraoptic nucleus around parturition. *J Neurosci* **23,** 788-797.

Kopp C, Rudolph U, Low K & Tobler I. (2004). Modulation of rhythmic brain activity by diazepam: $GABA_A$ receptor subtype and state specificity. *Proc Natl Acad Sci U S A* **101,** 3674-3679.

Korpi ER, Grunder G & Luddens H. (2002). Drug interactions at $GABA_A$ receptors. *Prog Neurobiol* **67,** 113-159.

Kralic JE, O'Buckley TK, Khisti RT, Hodge CW, Homanics GE & Morrow AL. (2002). $GABA_A$ receptor alpha-1 subunit deletion alters receptor subtype assembly, pharmacological and behavioral responses to benzodiazepines and zolpidem. *Neuropharmacology* **43,** 685-694.

Krishnan V & Nestler EJ. (2008). The molecular neurobiology of depression. *Nature* **455,** 894-902.

Kugaya A, Sanacora G, Verhoeff NP, Fujita M, Mason GF, Seneca NM, Bozkurt A, Khan SA, Anand A, Degen K, Charney DS, Zoghbi SS, Baldwin RM, Seibyl JP & Innis RB. (2003). Cerebral benzodiazepine receptors in depressed patients measured with [^{123}I]iomazenil SPECT. *Biol Psychiatry* **54,** 792-799.

Laakmann G, Faltermaier-Temizel M, Bossert-Zaudig S & Baghai T. (1996). Are benzodiazepines antidepressants? *Psychopharmacology (Berl)* **124,** 291-292.

Lacasse JR & Leo J. (2005). Serotonin and depression: a disconnect between the advertisements and the scientific literature. *PLoS Med* **2,** e392.

Lambert JJ, Belelli D, Peden DR, Vardy AW & Peters JA. (2003). Neurosteroid modulation of $GABA_A$ receptors. *Prog Neurobiol* **71,** 67-80.

Laurie DJ, Seeburg PH & Wisden W. (1992a). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. II. Olfactory bulb and cerebellum. *J Neurosci* **12,** 1063-1076.

Laurie DJ, Wisden W & Seeburg PH. (1992b). The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* **12,** 4151-4172.

Li GD, Chiara DC, Cohen JB & Olsen RW. (2009). Neurosteroids allosterically modulate binding of the anesthetic etomidate to γ-aminobutyric acid type A receptors. *J Biol Chem* **284,** 11771-11775.

Li P, Shu HJ, Wang C, Mennerick S, Zorumski CF, Covey DF, Steinbach JH & Akk G. (2007). Neurosteroid migration to intracellular compartments reduces steroid concentration in the membrane and diminishes $GABA_A$ receptor potentiation. *J Physiol* **584,** 789-800.

Lippa A, Czobor P, Stark J, Beer B, Kostakis E, Gravielle M, Bandyopadhyay S, Russek SJ, Gibbs TT, Farb DH & Skolnick P. (2005). Selective anxiolysis produced by ocinaplon, a $GABA_A$ receptor modulator. *Proc Natl Acad Sci U S A* **102,** 7380-7385.

Lister RG. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)* **92,** 180-185.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H & Rudolph U. (2000). Molecular and neuronal substrate for the selective attenuation of anxiety. *Science* **290,** 131-134.

Luisi S, Petraglia F, Benedetto C, Nappi RE, Bernardi F, Fadalti M, Reis FM, Luisi M & Genazzani AR. (2000). Serum allopregnanolone levels in pregnant women: changes during pregnancy, at delivery, and in hypertensive patients. *J Clin Endocrinol Metab* **85,** 2429-2433.

Luscher B, Fuchs T & Kilpatrick CL. (2011a). $GABA_A$ receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron* **70,** 385-409.

Luscher B & Keller CA. (2004). Regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. *Pharmacol Ther* **102,** 195-221.

Luscher B, Shen Q & Sahir N. (2011b). The GABAergic deficit hypothesis of major depressive disorder. *Mol Psychiatry* **16,** 383-406.

Luscher C & Ungless MA. (2006). The mechanistic classification of addictive drugs. *PLoS Med* **3,** e437.

MacQueen G & Frodl T. (2011). The hippocampus in major depression: evidence for the convergence of the bench and bedside in psychiatric research? *Mol Psychiatry* **16,** 252-264.

Maguire J & Mody I. (2008). $GABA_AR$ plasticity during pregnancy: relevance to postpartum depression. *Neuron* **59,** 207-213.

Maguire JL, Stell BM, Rafizadeh M & Mody I. (2005). Ovarian cycle-linked changes in $GABA_A$ receptors mediating tonic inhibition alter seizure susceptibility and anxiety. *Nat Neurosci* **8,** 797-804.

McCabe C, Cowen PJ & Harmer CJ. (2009). Neural representation of reward in recovered depressed patients. *Psychopharmacology (Berl)* **205,** 667-677.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR & Whiting PJ. (2000). Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor $\alpha 1$ subtype. *Nat Neurosci* **3,** 587-592.

McKernan RM & Whiting PJ. (1996). Which $GABA_A$ receptor subtypes really occur in the brain? *Trends Neurosci* **19,** 139-143.

Mellon SH, Gong W & Schonemann MD. (2008). Endogenous and synthetic neurosteroids in treatment of Niemann-Pick Type C disease. *Brain Res Rev* **57,** 410-420.

Mellon SH & Griffin LD. (2002). Neurosteroids: biochemistry and clinical significance. *Trends Endocrinol Metab* **13,** 35-43.

Mendelson WB, Martin JV, Perlis M, Wagner R, Majewska MD & Paul SM. (1987). Sleep induction by an adrenal steroid in the rat. *Psychopharmacology (Berl)* **93,** 226-229.

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW & Homanics GE. (1999). Attenuated sensitivity to neuroactive steroids in γ-aminobutyrate type A receptor delta subunit knockout mice. *Proc Natl Acad Sci U S A* **96,** 12905-12910.

Miller PS & Smart TG. (2010). Binding, activation and modulation of Cys-loop receptors. *Trends Pharmacol Sci* **31,** 161-174.

Mizokami A, Kanematsu T, Ishibashi H, Yamaguchi T, Tanida I, Takenaka K, Nakayama KI, Fukami K, Takenawa T, Kominami E, Moss SJ, Yamamoto T, Nabekura J & Hirata M. (2007). Phospholipase C-related inactive protein is involved in trafficking of γ2 subunit-containing $GABA_A$ receptors to the cell surface. *J Neurosci* **27,** 1692-1701.

Mody I. (2001). Distinguishing between $GABA_A$ receptors responsible for tonic and phasic conductances. *Neurochem Res* **26,** 907-913.

Mody I & Maguire J. (2011). The reciprocal regulation of stress hormones and $GABA_A$ receptors. *Front Cell Neurosci* **6,** 4.

Mohler H. (2006a). $GABA_A$ receptor diversity and pharmacology. *Cell Tissue Res* **326,** 505-516.

Mohler H. (2006b). $GABA_A$ receptors in central nervous system disease: anxiety, epilepsy, and insomnia. *J Recept Signal Transduct Res* **26,** 731-740.

Mohler H. (2011). The rise of a new GABA pharmacology. *Neuropharmacology* **60,** 1042-1049.

Mombereau C, Kaupmann K, Froestl W, Sansig G, van der Putten H & Cryan JF. (2004). Genetic and pharmacological evidence of a role for $GABA_B$ receptors in the modulation of anxiety- and antidepressant-like behavior. *Neuropsychopharmacology* **29,** 1050-1062.

Mombereau C, Kaupmann K, Gassmann M, Bettler B, van der Putten H & Cryan JF. (2005). Altered anxiety and depression-related behaviour in mice lacking $GABA_{B(2)}$ receptor subunits. *Neuroreport* **16,** 307-310.

Morris HV, Dawson GR, Reynolds DS, Atack JR, Rosahl TW & Stephens DN. (2008). Alpha2-containing $GABA_A$ receptors are involved in mediating stimulant effects of cocaine. *Pharmacol Biochem Behav* **90,** 9-18.

Mortensen M & Smart TG. (2006). Extrasynaptic $\alpha\beta$ subunit $GABA_A$ receptors on rat hippocampal pyramidal neurons. *J Physiol* **577,** 841-856.

Moss SJ & Smart TG. (2001). Constructing inhibitory synapses. *Nat Rev Neurosci* **2,** 240-250.

Mtchedlishvili Z, Sun CS, Harrison MB & Kapur J. (2003). Increased neurosteroid sensitivity of hippocampal $GABA_A$ receptors during postnatal development. *Neuroscience* **118,** 655-666.

Munzel U & Hothorn LA. (2001). A unified approach to simultaneous rank test procedures in the unbalanced one-way layout. *Biometrical Journal* **43,** 553-569.

Nguyen PN, Billiards SS, Walker DW & Hirst JJ. (2003). Changes in 5α-pregnane steroids and neurosteroidogenic enzyme expression in the perinatal sheep. *Pediatr Res* **53,** 956-964.

Nohria V & Giller E. (2007). Ganaxolone. *Neurotherapeutics* **4,** 102-105.

Nusser Z & Mody I. (2002). Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol* **87,** 2624-2628.

Nusser Z, Sieghart W, Benke D, Fritschy JM & Somogyi P. (1996). Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells. *Proc Natl Acad Sci U S A* **93,** 11939-11944.

Nusser Z, Sieghart W & Somogyi P. (1998). Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* **18,** 1693-1703.

Nutt D, Argyropoulos S, Hood S & Potokar J. (2006). Generalized anxiety disorder: A comorbid disease. *Eur Neuropsychopharmacol* **16 Suppl 2,** S109-118.

Nyiri G, Freund TF & Somogyi P. (2001). Input-dependent synaptic targeting of α2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat. *Eur J Neurosci* **13,** 428-442.

O'Brien CP. (2005). Benzodiazepine use, abuse, and dependence. *J Clin Psychiatry* **66 Suppl 2,** 28-33.

Okada M, Onodera K, Van Renterghem C, Sieghart W & Takahashi T. (2000). Functional correlation of $GABA_A$ receptor α subunits expression with the properties of IPSCs in the developing thalamus. *J Neurosci* **20,** 2202-2208.

Olsen RW & Li GD. (2011). $GABA_A$ receptors as molecular targets of general anesthetics: identification of binding sites provides clues to allosteric modulation. *Can J Anaesth* **58,** 206-215.

Otis TS & Mody I. (1992). Modulation of decay kinetics and frequency of $GABA_A$ receptor-mediated spontaneous inhibitory postsynaptic currents in hippocampal neurons. *Neuroscience* **49,** 13-32.

Overstreet LS, Jones MV & Westbrook GL. (2000). Slow desensitization regulates the availability of synaptic $GABA_A$ receptors. *J Neurosci* **20,** 7914-7921.

Pan ZH, Zhang X & Lipton SA. (2000). Redox modulation of recombinant human $GABA_A$ receptors. *Neuroscience* **98,** 333-338.

Papadopoulos V & Lecanu L. (2009). Translocator protein (18 kDa) TSPO: an emerging therapeutic target in neurotrauma. *Exp Neurol* **219,** 53-57.

Papp M, Willner P & Muscat R. (1991). An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. *Psychopharmacology (Berl)* **104,** 255-259.

Paul SM & Purdy RH. (1992). Neuroactive steroids. *FASEB J* **6,** 2311-2322.

Pellow S, Chopin P, File SE & Briley M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* **14,** 149-167.

Pellow S & File SE. (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. *Pharmacol Biochem Behav* **24,** 525-529.

Petty F. (1994). Plasma concentrations of γ-aminobutyric acid (GABA) and mood disorders: a blood test for manic depressive disease? *Clin Chem* **40,** 296-302.

Pham X, Sun C, Chen X, van den Oord EJ, Neale MC, Kendler KS & Hettema JM. (2009). Association study between GABA receptor genes and anxiety spectrum disorders. *Depress Anxiety* **26,** 998-1003.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W & Sperk G. (2000). $GABA_A$ receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. *Neuroscience* **101,** 815-850.

Poisbeau P, Patte-Mensah C, Keller AF, Barrot M, Breton JD, Luis-Delgado OE, Freund-Mercier MJ, Mensah-Nyagan AG & Schlichter R. (2005). Inflammatory

pain upregulates spinal inhibition via endogenous neurosteroid production. *J Neurosci* **25,** 11768-11776.

Porcu P, Sogliano C, Cinus M, Purdy RH, Biggio G & Concas A. (2003). Nicotine-induced changes in cerebrocortical neuroactive steroids and plasma corticosterone concentrations in the rat. *Pharmacol Biochem Behav* **74,** 683-690.

Porsolt RD, Bertin A & Jalfre M. (1978). "Behavioural despair" in rats and mice: strain differences and the effects of imipramine. *Eur J Pharmacol* **51,** 291-294.

Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy JM & Vogt KE. (2006). Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. *J Neurophysiol* **96,** 846-857.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR & Seeburg PH. (1989). Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature* **338,** 582-585.

Prys-Roberts C & Sear J. (1980). Steroid anaesthesia. *Br J Anaesth* **52,** 363-365.

Puia G, Mienville JM, Matsumoto K, Takahata H, Watanabe H, Costa E & Guidotti A. (2003). On the putative physiological role of allopregnanolone on $GABA_A$ receptor function. *Neuropharmacology* **44,** 49-55.

Puia G, Santi MR, Vicini S, Pritchett DB, Purdy RH, Paul SM, Seeburg PH & Costa E. (1990). Neurosteroids act on recombinant human $GABA_A$ receptors. *Neuron* **4,** 759-765.

Purdy RH, Moore PH, Jr., Rao PN, Hagino N, Yamaguchi T, Schmidt P, Rubinow DR, Morrow AL & Paul SM. (1990a). Radioimmunoassay of 3α-hydroxy-5α-pregnan-20-one in rat and human plasma. *Steroids* **55,** 290-296.

Purdy RH, Morrow AL, Blinn JR & Paul SM. (1990b). Synthesis, metabolism, and pharmacological activity of 3α-hydroxy steroids which potentiate GABA-receptor-mediated chloride ion uptake in rat cerebral cortical synaptoneurosomes. *J Med Chem* **33,** 1572-1581.

Purdy RH, Morrow AL, Moore PH, Jr. & Paul SM. (1991). Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. *Proc Natl Acad Sci U S A* **88,** 4553-4557.

Ramos B, Lopez-Tellez JF, Vela J, Baglietto-Vargas D, del Rio JC, Ruano D, Gutierrez A & Vitorica J. (2004). Expression of α5 $GABA_A$ receptor subunit in developing rat hippocampus. *Brain Res Dev Brain Res* **151,** 87-98.

Reddy DS & Rogawski MA. (2000). Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. *J Pharmacol Exp Ther* **295,** 1241-1248.

Reddy DS & Rogawski MA. (2009). Neurosteroid replacement therapy for catamenial epilepsy. *Neurotherapeutics* **6,** 392-401.

Reddy DS & Rogawski MA. (2010). Ganaxolone suppression of behavioral and electrographic seizures in the mouse amygdala kindling model. *Epilepsy Res* **89,** 254-260.

Reith CA & Sillar KT. (1997). Pre- and postsynaptic modulation of spinal GABAergic neurotransmission by the neurosteroid, 5β-pregnan-3α-ol-20-one. *Brain Res* **770,** 202-212.

Reynolds DS, McKernan RM & Dawson GR. (2001). Anxiolytic-like action of diazepam: which $GABA_A$ receptor subtype is involved? *Trends Pharmacol Sci* **22,** 402-403.

Reynolds LM, Engin E, Tantillo G, Lau HM, Muschamp JW, Carlezon WA, Jr. & Rudolph U. (2012). Differential roles of $GABA_A$ receptor subtypes in benzodiazepine-induced enhancement of brain-stimulation reward. *Neuropsychopharmacology* **37,** 2531-2540.

Richerson GB. (2004). Looking for GABA in all the wrong places: the relevance of extrasynaptic $GABA_A$ receptors to epilepsy. *Epilepsy Curr* **4,** 239-242.

Rodgers RJ & Johnson NJ. (1995). Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. *Pharmacol Biochem Behav* **52,** 297-303.

Rodgers RJ & Johnson NJ. (1998). Behaviorally selective effects of neuroactive steroids on plus-maze anxiety in mice. *Pharmacol Biochem Behav* **59,** 221-232.

Romeo E, Strohle A, Spalletta G, di Michele F, Hermann B, Holsboer F, Pasini A & Rupprecht R. (1998). Effects of antidepressant treatment on neuroactive steroids in major depression. *Am J Psychiatry* **155,** 910-913.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H & Mohler H. (1999). Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. *Nature* **401,** 796-800.

Ruiz A, Walker MC, Fabian-Fine R & Kullmann DM. (2004). Endogenous Zinc inhibits $GABA_A$ receptors in a hippocampal pathway. *J Neurophysiol* **91,** 1091-1096.

Rupprecht R & Holsboer F. (1999). Neuroactive steroids: mechanisms of action and neuropsychopharmacological perspectives. *Trends Neurosci* **22,** 410-416.

Saalmann YB, Kirkcaldie MT, Waldron S & Calford MB. (2007). Cellular distribution of the $GABA_A$ receptor-modulating 3α-hydroxy, 5α-reduced pregnane steroids in the adult rat brain. *J Neuroendocrinol* **19,** 272-284.

Sanacora G, Mason GF, Rothman DL, Behar KL, Hyder F, Petroff OA, Berman RM, Charney DS & Krystal JH. (1999). Reduced cortical γ-aminobutyric acid levels in depressed patients determined by proton magnetic resonance spectroscopy. *Arch Gen Psychiatry* **56,** 1043-1047.

Santi MR, Vicini S, Eldadah B & Neale JH. (1994). Analysis by polymerase chain reaction of α1 and α6 $GABA_A$ receptor subunit mRNAs in individual cerebellar neurons after whole-cell recordings. *J Neurochem* **63,** 2357-2360.

Sarviharju M, Hyytia P, Hervonen A, Jaatinen P, Kiianmaa K & Korpi ER. (2006). Lifelong ethanol consumption and brain regional $GABA_A$ receptor subunit mRNA expression in alcohol-preferring rats. *Alcohol* **40,** 159-166.

Scimemi A, Semyanov A, Sperk G, Kullmann DM & Walker MC. (2005). Multiple and plastic receptors mediate tonic $GABA_A$ receptor currents in the hippocampus. *J Neurosci* **25,** 10016-10024.

Semyanov A, Walker MC & Kullmann DM. (2003). GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. *Nat Neurosci* **6,** 484-490.

Semyanov A, Walker MC, Kullmann DM & Silver RA. (2004). Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. *Trends Neurosci* **27,** 262-269.

Serra M, Pisul MG, Dazzi L, Purdy RH & Biggio G. (2002). Prevention of the stress-induced increase in the concentration of neuroactive steroids in rat brain by long-term administration of mirtazapine but not of fluoxetine. *J Psychopharmacol* **16,** 133-138.

Sheline YI. (2003). Neuroimaging studies of mood disorder effects on the brain. *Biol Psychiatry* **54,** 338-352.

Shen H, Gong QH, Aoki C, Yuan M, Ruderman Y, Dattilo M, Williams K & Smith SS. (2007). Reversal of neurosteroid effects at $\alpha4\beta2\delta$ $GABA_A$ receptors triggers anxiety at puberty. *Nat Neurosci* **10,** 469-477.

Shen Q, Lal R, Luellen BA, Earnheart JC, Andrews AM & Luscher B. (2010). γ-aminobutyric acid-type A receptor deficits cause hypothalamic-pituitary-adrenal axis hyperactivity and antidepressant drug sensitivity reminiscent of melancholic forms of depression. *Biol Psychiatry* **68,** 512-520.

Shirayama Y & Chaki S. (2006). Neurochemistry of the nucleus accumbens and its relevance to depression and antidepressant action in rodents. *Curr Neuropharmacol* **4,** 277-291.

Shirayama Y, Muneoka K, Fukumoto M, Tadokoro S, Fukami G, Hashimoto K & Iyo M. (2011). Infusions of allopregnanolone into the hippocampus and amygdala, but not into the nucleus accumbens and medial prefrontal cortex, produce antidepressant effects on the learned helplessness rats. *Hippocampus* **21,** 1105-1113.

Sieghart W & Sperk G. (2002). Subunit composition, distribution and function of $GABA_A$ receptor subtypes. *Curr Top Med Chem* **2,** 795-816.

Smart TG. (1997). Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. *Curr Opin Neurobiol* **7,** 358-367.

Smart TG, Hosie AM & Miller PS. (2004). Zn^{2+} ions: modulators of excitatory and inhibitory synaptic activity. *Neuroscientist* **10,** 432-442.

Smith KS & Rudolph U. (2012). Anxiety and depression: mouse genetics and pharmacological approaches to the role of $GABA_A$ receptor subtypes. *Neuropharmacology* **62,** 54-62.

Smith SS, Gong QH, Hsu FC, Markowitz RS, ffrench-Mullen JM & Li X. (1998). $GABA_A$ receptor α4 subunit suppression prevents withdrawal properties of an endogenous steroid. *Nature* **392,** 926-930.

Somogyi P, Takagi H, Richards JG & Mohler H. (1989). Subcellular localization of benzodiazepine/$GABA_A$ receptors in the cerebellum of rat, cat, and monkey using monoclonal antibodies. *J Neurosci* **9,** 2197-2209.

Song M & Messing RO. (2005). Protein kinase C regulation of $GABA_A$ receptors. *Cell Mol Life Sci* **62,** 119-127.

Sperk G, Schwarzer C, Tsunashima K, Fuchs K & Sieghart W. (1997). $GABA_A$ receptor subunits in the rat hippocampus I: Immunocytochemical distribution of 13 subunits. *Neuroscience* **80,** 987-1000.

Stanford SC. (2007). The open field test: reinventing the wheel. *J Psychopharmacol* **21,** 134-135.

Steland A, Padmanabhan AR & Akram M. (2011). Resampling methods for the nonparametric and generalised Behrens-Fisher problems. *Indian J Statistics* **73-A,** 267-302.

Stell BM, Brickley SG, Tang CY, Farrant M & Mody I. (2003). Neuroactive steroids reduce neuronal excitability by selectively enhancing tonic inhibition mediated

by δ subunit-containing $GABA_A$ receptors. *Proc Natl Acad Sci U S A* **100,** 14439-14444.

Steru L, Chermat R, Thierry B & Simon P. (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* **85,** 367-370.

Stocco DM & Clark BJ. (1996). Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis. *Biochem Pharmacol* **51,** 197-205.

Stoffel-Wagner B. (2003). Neurosteroid biosynthesis in the human brain and its clinical implications. *Ann N Y Acad Sci* **1007,** 64-78.

Strohle A, Pasini A, Romeo E, Hermann B, Spalletta G, di Michele F, Holsboer F & Rupprecht R. (2000). Fluoxetine decreases concentrations of 3α,5α-tetra-hydro-deoxy-corticosterone (THDOC) in major depression. *J Psychiatr Res* **34,** 183-186.

Strohle A, Romeo E, Hermann B, Pasini A, Spalletta G, di Michele F, Holsboer F & Rupprecht R. (1999). Concentrations of 3α-reduced neuroactive steroids and their precursors in plasma of patients with major depression and after clinical recovery. *Biol Psychiatry* **45,** 274-277.

Strous RD, Maayan R & Weizman A. (2006). The relevance of neurosteroids to clinical psychiatry: from the laboratory to the bedside. *Eur Neuropsychopharmacol* **16,** 155-169.

Szabadics J, Varga C, Molnar G, Olah S, Barzo P & Tamas G. (2006). Excitatory effect of GABAergic axo-axonic cells in cortical microcircuits. *Science* **311,** 233-235.

Tan KR, Brown M, Labouebe G, Yvon C, Creton C, Fritschy JM, Rudolph U & Luscher C. (2010). Neural bases for addictive properties of benzodiazepines. *Nature* **463,** 769-774.

Tan KR, Rudolph U & Luscher C. (2011). Hooked on benzodiazepines: $GABA_A$ receptor subtypes and addiction. *Trends Neurosci* **34,** 188-197.

Thierry B, Steru L, Simon P & Porsolt RD. (1986). The tail suspension test: ethical considerations. *Psychopharmacology (Berl)* **90,** 284-285.

Thomas P, Mortensen M, Hosie AM & Smart TG. (2005). Dynamic mobility of functional $GABA_A$ receptors at inhibitory synapses. *Nat Neurosci* **8,** 889-897.

Thomas P & Smart TG. (2005). HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods* **51,** 187-200.

Thompson SA, Whiting PJ & Wafford KA. (1996). Barbiturate interactions at the human $GABA_A$ receptor: dependence on receptor subunit combination. *Br J Pharmacol* **117,** 521-527.

Tobler I, Kopp C, Deboer T & Rudolph U. (2001). Diazepam-induced changes in sleep: role of the α1 $GABA_A$ receptor subtype. *Proc Natl Acad Sci U S A* **98,** 6464-6469.

Trapani G, Franco M, Ricciardi L, Latrofa A, Genchi G, Sanna E, Tuveri F, Cagetti E, Biggio G & Liso G. (1997). Synthesis and binding affinity of 2-phenylimidazo[1,2-α]pyridine derivatives for both central and peripheral benzodiazepine receptors. A new series of high-affinity and selective ligands for the peripheral type. *J Med Chem* **40,** 3109-3118.

Tretter V, Ehya N, Fuchs K & Sieghart W. (1997). Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. *J Neurosci* **17,** 2728-2737.

Tretter V, Jacob TC, Mukherjee J, Fritschy JM, Pangalos MN & Moss SJ. (2008). The clustering of $GABA_A$ receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor α2 subunits to gephyrin. *J Neurosci* **28,** 1356-1365.

Tretter V, Kerschner B, Milenkovic I, Ramsden SL, Ramerstorfer J, Saiepour L, Maric HM, Moss SJ, Schindelin H, Harvey RJ, Sieghart W & Harvey K. (2011). Molecular basis of the γ-aminobutyric acid A receptor α3 subunit interaction with the clustering protein gephyrin. *J Biol Chem* **286,** 37702-37711.

Turkmen S, Backstrom T, Wahlstrom G, Andreen L & Johansson IM. (2011). Tolerance to allopregnanolone with focus on the GABA-A receptor. *Br J Pharmacol* **162,** 311-327.

Twelvetrees AE, Yuen EY, Arancibia-Carcamo IL, MacAskill AF, Rostaing P, Lumb MJ, Humbert S, Triller A, Saudou F, Yan Z & Kittler JT. (2010). Delivery of $GABA_A$Rs to synapses is mediated by HAP1-KIF5 and disrupted by mutant huntingtin. *Neuron* **65,** 53-65.

Uzunov DP, Cooper TB, Costa E & Guidotti A. (1996). Fluoxetine-elicited changes in brain neurosteroid content measured by negative ion mass fragmentography. *Proc Natl Acad Sci U S A* **93,** 12599-12604.

Uzunova V, Sheline Y, Davis JM, Rasmusson A, Uzunov DP, Costa E & Guidotti A. (1998). Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. *Proc Natl Acad Sci U S A* **95,** 3239-3244.

Vallee M, Rivera JD, Koob GF, Purdy RH & Fitzgerald RL. (2000). Quantification of neurosteroids in rat plasma and brain following swim stress and allopregnanolone administration using negative chemical ionization gas chromatography/mass spectrometry. *Anal Biochem* **287,** 153-166.

van der Heyden JA, Molewijk E & Olivier B. (1987). Strain differences in response to drugs in the tail suspension test for antidepressant activity. *Psychopharmacology (Berl)* **92,** 127-130.

van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM & Crestani F. (2004). Requirement of α5-$GABA_A$ receptors for the development of tolerance to the sedative action of diazepam in mice. *J Neurosci* **24,** 6785-6790.

VanDoren MJ, Matthews DB, Janis GC, Grobin AC, Devaud LL & Morrow AL. (2000). Neuroactive steroid 3α-hydroxy-5α-pregnan-20-one modulates electrophysiological and behavioral actions of ethanol. *J Neurosci* **20,** 1982-1989.

Vollenweider I, Smith KS, Keist R & Rudolph U. (2011). Antidepressant-like properties of α2-containing $GABA_A$ receptors. *Behav Brain Res* **217,** 77-80.

Wang H, Bedford FK, Brandon NJ, Moss SJ & Olsen RW. (1999). $GABA_A$-receptor-associated protein links $GABA_A$ receptors and the cytoskeleton. *Nature* **397,** 69-72.

Wang M, He Y, Eisenman LN, Fields C, Zeng CM, Mathews J, Benz A, Fu T, Zorumski E, Steinbach JH, Covey DF, Zorumski CF & Mennerick S. (2002). 3β-hydroxypregnane steroids are pregnenolone sulfate-like $GABA_A$ receptor antagonists. *J Neurosci* **22,** 3366-3375.

Whiting P, McKernan RM & Iversen LL. (1990). Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc Natl Acad Sci U S A* **87,** 9966-9970.

Wieland HA, Luddens H & Seeburg PH. (1992). A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. *J Biol Chem* **267,** 1426-1429.

Wieland S, Lan NC, Mirasedeghi S & Gee KW. (1991). Anxiolytic activity of the progesterone metabolite 5α-pregnan-3α-o1-20-one. *Brain Res* **565,** 263-268.

Wilkins ME, Hosie AM & Smart TG. (2002). Identification of a β subunit TM2 residue mediating proton modulation of GABA type A receptors. *J Neurosci* **22,** 5328-5333.

Wilkins ME, Hosie AM & Smart TG. (2005). Proton modulation of recombinant $GABA_A$ receptors: influence of GABA concentration and the β subunit TM2-TM3 domain. *J Physiol* **567,** 365-377.

Winter L, Nadeson R, Tucker AP & Goodchild CS. (2003). Antinociceptive properties of neurosteroids: a comparison of alphadolone and alphaxalone in potentiation of opioid antinociception. *Anesth Analg* **97,** 798-805.

Wisden W, Cope D, Klausberger T, Hauer B, Sinkkonen ST, Tretter V, Lujan R, Jones A, Korpi ER, Mody I, Sieghart W & Somogyi P. (2002). Ectopic expression of the $GABA_A$ receptor α6 subunit in hippocampal pyramidal neurons produces extrasynaptic receptors and an increased tonic inhibition. *Neuropharmacology* **43,** 530-549.

Wisden W, Laurie DJ, Monyer H & Seeburg PH. (1992). The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J Neurosci* **12,** 1040-1062.

Wisden W & Seeburg PH. (1992). $GABA_A$ receptor channels: from subunits to functional entities. *Curr Opin Neurobiol* **2,** 263-269.

Wu X, Wu Z, Ning G, Guo Y, Ali R, Macdonald RL, De Blas AL, Luscher B & Chen G. (2012). γ-aminobutyric acid type A ($GABA_A$) receptor α subunits play a direct role in synaptic versus extrasynaptic targeting. *J Biol Chem* **287,** 27417-27430.

Xi ZX, Ramamoorthy S, Shen H, Lake R, Samuvel DJ & Kalivas PW. (2003). GABA transmission in the nucleus accumbens is altered after withdrawal from repeated cocaine. *J Neurosci* **23,** 3498-3505.

Yamada K, Watanabe A, Iwayama-Shigeno Y & Yoshikawa T. (2003). Evidence of association between gamma-aminobutyric acid type A receptor genes located on 5q34 and female patients with mood disorders. *Neurosci Lett* **349,** 9-12.

Yee BK, Keist R, von Boehmer L, Studer R, Benke D, Hagenbuch N, Dong Y, Malenka RC, Fritschy JM, Bluethmann H, Feldon J, Mohler H & Rudolph U. (2005). A schizophrenia-related sensorimotor deficit links α3-containing $GABA_A$ receptors to a dopamine hyperfunction. *Proc Natl Acad Sci U S A* **102,** 17154-17159.

Zeilhofer HU. (2008). Loss of glycinergic and GABAergic inhibition in chronic pain - contributions of inflammation and microglia. *Int Immunopharmacol* **8,** 182-187.

Zeilhofer HU, Wildner H & Yevenes GE. (2012). Fast synaptic inhibition in spinal sensory processing and pain control. *Physiol Rev* **92,** 193-235.

Zhu WJ & Vicini S. (1997). Neurosteroid prolongs $GABA_A$ channel deactivation by altering kinetics of desensitized states. *J Neurosci* **17,** 4022-4031.